PUBLIC HEALTH
LAW CENTER
Tobacco Control Legal Consortium

July 16, 2018

Commissioner Scott Gottlieb, MD
c/o Division of Dockets Management
HFA-305
Food and Drug Administration
5630 Fishers Lane, Room 1061
Rockville, MD 20825

Re: Tobacco Product Standard for Nicotine Level of Combusted Cigarettes

Docket No. FDA-2017-N-6189

Dear Commissioner Gottlieb:

The Public Health Law Center is pleased to submit these comments to the U.S. Food and Drug Administration (FDA) on the potential regulation of nicotine levels in combustible tobacco products. The Public Health Law Center is the coordinating center of the Tobacco Control Legal Consortium, a national network of nonprofit legal centers providing legal technical assistance to public health professionals and advocates concerning legal issues related to tobacco and public health.[1]

There is no question that commercial tobacco use is the greatest public health crisis facing our country. Despite incredible progress made over the last sixty years, commercial tobacco use is still the largest cause of preventable death and disease in the U.S. Over 480,000 people die every year as a result of first or secondhand tobacco exposure. By far, the largest portion of that death and disease is attributable to cigarettes and other combustible tobacco products. Taking nicotine out of the equation should dramatically reduce tobacco use. Disincentivizing the use of combustible tobacco products has been the goal of many tobacco control policies that have been initiated by all levels of government. Never before has any government attempted such a bold step as targeting the very agent that creates and

[1] The Tobacco Control Legal Consortium's activities are coordinated by the Public Health Law Center at Mitchell Hamline School of Law in St. Paul, Minnesota. The Consortium's affiliated legal centers include: ChangeLab Solutions, Oakland, California; Legal Resource Center for Tobacco Regulation, Litigation & Advocacy, at University of Maryland Francis King Carey School of Law, Baltimore, Maryland; Public Health Advocacy Institute and the Center for Public Health and Tobacco Policy, both at Northeastern University School of Law, Boston, Massachusetts; Smoke-Free Environments Law Project, at the University of Michigan, Ann Arbor, Michigan; and Tobacco Control Policy and Legal Resource Center at New Jersey GASP, Summit, New Jersey.

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sustains addiction to tobacco products. Such a step should bring about a seismic shift in the commercial tobacco epidemic. We strongly support the FDA's action and recommend that the agency make the rule as strong as is possible in order to maximize the public health benefits of such a rule.

We encourage the FDA to propose a truly comprehensive nicotine reduction rule. First and foremost, a rule reducing nicotine levels in any tobacco products must apply to all combustible tobacco products, not just cigarettes. Second, the rule must reduce nicotine to a level that maximizes the public health benefits. These measures would be best implemented through an immediate one-time reduction in nicotine for combustible products, followed by a step-down reduction in nicotine for non-combustible products to finally break the cycle of nicotine addiction for all tobacco product users. Furthermore, the agency will achieve the greatest public health benefit by simultaneously implementing an educational campaign to explain the effects of nicotine reduction, highlighting the fact that, while combustible products are no longer addictive, they are certainly still deadly.

Finally, the opportunity presented by a nicotine product standard to dramatically increase cessation requires several related agency actions to be fully realized. The FDA must take steps to increase access to and innovation of nicotine replacement therapies. The agency also needs to establish product standards that will make non-combustible products safer. The FDA must also use its specific authority under the Tobacco Control Act to establish a track-and-trace system to mitigate the potential for illicit trade to undermine the public health benefits of such a rule. Finally, the agency must take any other necessary steps to ensure that the tobacco industry is unable to undermine the potential benefits of the rule.

I. A Product Standard for Nicotine in Tobacco Products Must Be Comprehensive.

With a mandate to protect public health, the FDA must design a product standard for nicotine that eliminates addiction and maximizes cessation. The scope of the commercial tobacco epidemic in the U.S. warrants comprehensive agency action that is the most protective of public health.

Swift action is also warranted. The agency has faced litigation challenging nearly all of its past efforts to regulate tobacco products. This industry tactic delays the regulatory process at every stage. No amount of agency caution can forestall the inevitable industry lawsuits.

a. A Product Standard for Nicotine Must Apply to All Commercial Combustible Tobacco Products.

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While many tobacco control policies have rightly focused on the impact of cigarettes as the most significant disease vector in tobacco control, in taking a robust approach to nicotine reduction, the FDA must recognize that all tobacco products that partially combust cured, processed tobacco, as cigarettes do, have the potential to be equally toxic and carcinogenic. Cigarettes have historically been the largest cause of public health harm because they are the most popular combustible tobacco product. However, despite minor differences in curing and processing methods, almost all commercial tobacco products utilize the same tobacco plant species, *Nicotiana tabacum*.[2] Consequently, the tobacco in cigarettes is fundamentally the same as the tobacco used in cigars, pipe tobacco, roll-your-own tobacco, and shisha/waterpipe tobacco, creating the same types of health harms.[3] These health harms are also likely present in contemporary products that also rely on the imperfect combustion of processed, cured tobacco leaf (for example, heated cigarettes like Phillip Morris International's iQOS). In addition, if the FDA were to establish a product standard that covers only cigarettes or only some combustible tobacco products, we know from past practices that the tobacco industry would quickly exploit such a loophole to maximize its profits through the sale of combustible products that are not subject to a nicotine product standard but have significant or indistinguishable health harms.

i. The Health Harms of Different Classes of Combustible Products Are the Same or Similar.

While there may be some varying levels of exposure to particular toxicants or carcinogens between different types of combustible tobacco products, ultimately, the level of risk of harm between these products is not distinguishable. When compared to cigarettes, the severity of the potential negative health outcomes combined with the probability of developing those outcomes are not distinguishably less for other types of combustible tobacco products. Two of the main reasons that these products still present significant and severe health risks is that they are 1) comprised of processed tobacco leaf and 2) partially or imperfectly combusted. This is not surprising given the fact that the tobacco in these products is essentially the same.[4]

The processing of tobacco leaf through curing and fermentation play an important role in determining the level of toxicants in tobacco and its smoke. Typically, tobacco carcinogens referred to as tobacco-specific nitrosamines (TSNAs), including the most carcinogenic N'-nitrosonornicotine, NNN, and 4-(methylnitrosamino)-1-(3-

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[2] WORLD HEALTH ORG. INT'L AGENCY FOR RESEARCH ON CANCER, *Tobacco Smoke and Involuntary Smoking*, 83 IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS (2004), at 58.
[3] *Id.* at 58-59.
[4] See e.g. TRP: TOBACCO RAG PROCESSORS, INC., http://tobaccorag.com/products/cut-rag (last visited July 10, 2018).

pyridyl)-1-butanone, NNK, are created through the processing of tobacco leaf.[5]. In several studies, the levels of NNN and NNK in cigars and little cigars are equal to or exceed the levels in combustible cigarettes.[6]

Polycyclic Aromatic Hydrocarbons or PAHs are formed when any organic matter is not completely burned.[7] This process is often referred to as imperfect combustion. Many PAHs are highly volatile and present significant risks to both smokers and those who are exposed to secondhand smoke. While not much research has focused specifically on little cigars, at least one study has found that with both human testers and automated smoking machines, the levels of PAHs in little cigars are similar or greater than the level of PAHs in cigarette smoke.[8] Additionally, in at least one environmental study of the level of secondhand PAH exposure, the total levels of PAH emissions were greater for cigar social events than for comparable cigarette measures.[9]

When comparing particulate matter from cigars and cigarillos to cigarette smoke, at least one study found that the cigar and cigarillo matter was 200% more mutagenic, or more capable of damaging the user's DNA, per unit of nicotine.[10] In fact, smokers who switch to cigar smoking have little benefit in terms of mortality.[11] It is also important to note that a significant percentage of tobacco users believe that cigars and little cigars are less harmful than combustible cigarettes.[12] FDA action on nicotine levels that exempts any type of cigars could potentially reinforce this misbelief and encourage cigarette smokers to switch to these equally harmful products.

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[5] *See* Buddy G. Brown et al., *An Analysis of the Role of Tobacco-Specific Nitrosamines in the Carcinogenicity of Tobacco Smoke*, 1 NONLINEARITY IN BIOLOGY, TOXICOLOGY, AND MED. 179 (2003). Samera H. Hamad et al., *Little Cigars vs 3R4F Cigarette: Physical Properties and HPHC Yields*, 3 TOBACCO REG. SCI. 459 (2017).

[7] U.S. DEP'T OF HEALTH AND HUMAN SERV., HOW TOBACCO SMOKE CAUSES DISEASE: THE BIOLOGY AND BEHAVIORAL BASIS FOR SMOKING-ATTRIBUTABLE DISEASE: A REPORT OF THE SURGEON GENERAL 37 (2010).

[8] Bartosz Koszowski et al., *Polycyclic Aromatic Hydrocarbons Levels in Mainstream Smoke of Little Cigars and Cigarettes Differ for ISO and Human Smoking* (SRNT Annual Meeting 2015).

[9] Neil E. Klepeis et al., *The Effect of Cigar Smoking on Indoor Levels of Carbon Monoxide and Particles*, 9 J. OF EXPOSURE SCI. & ENVTL. EPIDEMIOLOGY 622 (1999).

[10] William S. Rickert et al., *A Comparative Study of the Mutagenicity of Various Types of Tobacco Products*, 48 REG. TOXICOLOGY AND PHARMACOLOGY 320 (2007).

[11] *See* Frank Baker et al., *Health Risks Associated with Cigar Smoking*, 284 JAMA 735 (2000). *See also* Nicholas J. Wald & Hilary C. Watt, *Prospective Study of Effect of Switching from Cigarettes to Pipes or Cigars on Mortality from Three Smoking Related Diseases*, 314 BMJ 1860 (1997).

[12] Ruth E. Malone et al., *Cigar Risk Perceptions in Focus Groups of Urban African American Youth*, 13 J. OF SUBSTANCE ABUSE 549 (2001); *see also, e.g.,* Kymberle L. Sterling et al., *Risk Perceptions of Little Cigar and Cigarillo Smoking Among Adult Current Cigarette Smokers*, 19 NICOTINE & TOBACCO RES. 1351 (2016); Frank Baker et al., *Risk Perception and Cigar Smoking Behavior*, 25 AM. J. HEALTH BEHAV. 106 (2001).

ii. Exempting Any Class of Combustible Tobacco Invites Loophole Exploitation.

Because the health harms of all combustible products are indistinguishable, if the FDA were to establish a nicotine standard only for cigarettes or if such a standard left any combustible products with a level of nicotine that can create and sustain addiction, the public health benefits of a rule would be significantly compromised.

One need not look very far into the past to find examples of tobacco companies exploiting regulations that cover some products and not others. Consider the interplay of two important tobacco control measures, the Tobacco Control Act and the Children's Health Insurance Program Reauthorization Act of 2009 (CHIPRA). The Tobacco Control Act placed stringent restrictions on the manufacture and sale of cigarettes, prohibiting flavors other than menthol and tobacco,[13] prohibiting the use of modified risk terms including "light" and "low tar,"[14] and imposing significant advertising and marketing restrictions.[15] However, the Tobacco Control Act deferred any regulation of cigars to the future.[16] CHIPRA increased federal excise taxes on all tobacco products but did not increase taxes equitably, leaving the tax on cigars lower than the tax on cigarettes.[17] The result of these two actions was that many manufacturers of discount cigarettes slightly modified their products, by adding a nominal amount of tobacco to the paper wrappers, converting the cigarettes into cigars for regulatory and taxation purposes.[18] These new brands of cigars, often referred to as "little cigars," are functionally identical to cigarettes. They are sold in a variety of flavors; they use prohibited terms for cigarettes like "low" and "mild;" and the products are subject to a lower federal tax.[19] Manufacturers of roll-your-own tobacco found themselves in a similar position and simply changed the labeling on their products to "pipe tobacco," a product not immediately subject to FDA authority and also taxed at a lower rate than roll-your-own tobacco.[20] Cigar manufacturers were also able to exploit CHIPRA by converting "small cigars" to "large cigars" by adding sepiolite[21] to increase cigar weight, moving

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[13] Family Smoking Prevention and Tobacco Control Act, 21 U.S.C. § 387g(a)(1)(A) (2012).
[14] *Id.* § 387k(b)(2)(A)(ii).
[15] *See, e.g.,* 21 C.F.R. § 1140.1 – 1140.34 (2018).
[16] Family Smoking Prevention and Tobacco Control Act, 21 U.S.C. § 387a(b) (2012).
[17] Children's Health Insurance Program Reauthorization Act of 2009, 42 U.S.C. § 1396 (2012).
[18] U.S. GOV'T ACCOUNTABILITY OFF., GAO-12-475, TOBACCO TAXES: LARGE DISPARITIES IN RATES FOR SMOKING PRODUCTS TRIGGER SIGNIFICANT MARKET SHIFTS TO AVOID HIGHER TAXES (2012), https://www.gao.gov/assets/600/590192.pdf.
[19] *Id.*
[20] *Id.* This particular change only required manufacturers to change their packaging, *see id*. https://www.gao.gov/assets/600/590192.pdf
[21] Sepiolite is a clay substance that is also used in cat litter. Haydn H. Murray, *Traditional and New Applications for Kaolin, Smectite, and Palygorskite: A General Overview*, 17 APPLIED CLAY SCI. 207 (2000).

the products into a more favorable tax category.[22] This one change is estimated to have allowed cigar companies to avoid over $1 billion in taxes in the first four years following CHIPRA. The disparate regulation created an economic incentive to exploit the loophole. The result of the exploitation is that the use of the product categories that were not subject to the most stringent regulation or taxation went up, as consumers moved to less expensive and less stringently regulated products.[23]

There can be little doubt that a product standard for nicotine that only covers some combustible products will lead to similar industry behavior. The largest share of the tobacco product market is controlled by a small handful of companies who have been found to be in current, continuing violation of federal racketeering laws.[24] Even without deliberate industry manipulation, it is also likely that some cigarette smokers who have difficulty quitting would move to other combustible products with an addiction-sustaining level of nicotine. Many smokers have reported that they have not completely switched to e-cigarettes because the products do not fully replicate the experience of smoking cigarettes.[25] No similar barrier exists to switch to little cigars which are essentially identical to cigarettes with a small amount of tobacco in their paper wrapper. Even the FDA has recognized that many little cigar brands have simply converted cigarettes into cigars to escape stringent FDA regulation of cigarettes.[26]

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[22] Press release, Mathew L. Myers, President, Campaign for Tobacco-Free Kids, *Talk about a Scoop: Tobacco Company Puts Kitty Litter in Its Cigars* (March 1, 2013), https://www.tobaccofreekids.org/press-releases/2013_03_01_kittylitter; Anna Edny, Tobacco Firms Save $1 Billion With Kitty Litter in Cigars, Bloomberg (Feb. 28, 2013), https://www.bloomberg.com/news/articles/2013-03-01/tobacco-firms-save-1-billion-with-kitty-litter-in-cigars.

[23] Centers for Disease Control and Prevention, *Consumption of Cigarettes and Combustible Tobacco – United States, 2000-2011*, 61 MORBIDITY AND MORTALITY WKLY. REP. 30, 565 (2012), https://www.cdc.gov/mmwr/pdf/wk/mm6130.pdf.

[24] U.S. v. Philip Morris, Inc., 449 F. Supp. 2d 1 (D.D.C. 2006), *aff'd in relevant part*, 566 F.2d 1095 (D.C. Cir. 2009), *cert. denied*, 130 S.Ct. 3501 (2010).

[25] Jessica K. Pepper et al., *Reasons for Starting and Stopping Electronic Cigarette Use,* 11 INT'L J. OF ENVTL. RES. AND PUB. HEALTH 10, 10345 (2014), https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4210982/pdf/ijerph-11-10345.pdf.

[26] Letter from Ann Simoneau, Director, FDA Ctr. for Tobacco Prod., to Peter Ghiloni, President, Swisher International, Inc. (Dec. 9, 2016), https://www.fda.gov/iceci/enforcementactions/warningletters/2016/ucm558359.htm; Letter from Ann Simoneau, Director, FDA Ctr. for Tobacco Prod., to David A. Scott, CEO, Cheyenne Int'l, LLC (Dec. 9, 2016), https://www.fda.gov/iceci/enforcementactions/warningletters/2016/ucm532249.htm; Letter from Ann Simoneau, Director, FDA Ctr. for Tobacco Prod., to James L. Emery, CEO, Prime Time Int'l Distrib., (Dec. 9, 2016), https://www.fda.gov/iceci/enforcementactions/warningletters/2016/ucm532259.htm; Letter from Ann Simoneau, Director, FDA Ctr. for Tobacco Prod., to Richard L. Prezelski, President, S. Cross Tobacco Co., Inc., (Dec. 9, 2016), https://www.fda.gov/iceci/enforcementactions/warningletters/2016/ucm532265.htm.

Of all of the decisions that the FDA must make in implementing a product standard for nicotine, the decision of whether to cover all combustible products is perhaps the most crucial. A rule that is not comprehensive will not maximize the public health benefits and will unnecessarily cost lives.

b. A Product Standard for Nicotine in Combustible Products Must be a One-Time Reduction to a Level that Maximizes Public Health Benefits.

In addition to covering all combustible products, a nicotine standard for these products must be a one-time reduction that reduces nicotine to sub addictive levels.

There is clear evidence that a one-time reduction in nicotine will result in more quitting and greater decreases in consumption than a step-down approach. The use of cigarettes with very low nicotine content (yield of about 0.05 mg nicotine or less) reduces smoking and toxicant exposure more than cigarettes with intermediate or higher nicotine content. Very low nicotine content cigarette usage also results in less nicotine dependence and withdrawal symptoms, and is ultimately associated with quitting smoking. The intermediate nicotine levels required for a step-down approach are less effective in this regard. A single, very low level of nicotine significantly increases cessation over intermediate levels. [27] Additionally, when compared to a gradual approach to nicotine reduction, a single, immediate reduction results in greater reduction in: cigarettes smoked per day, nicotine exposure, NNK levels, Volatile Organic Compound (VOCs) exposure, and NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol) levels.[28] Furthermore, compensatory smoking behavior can potentially accompany intermediate nicotine levels, undermining the benefits they might otherwise have. At least one study found that the lowest levels of nicotine produced the best cessation outcomes.[29] As such, implementing a step-down nicotine reduction would not result in any benefits over a one-time reduction and would only prolong exposure to the harmful constituents of tobacco and tobacco smoke, putting many more lives at risk.

There is no public health justification to take a step-down approach when the evidence shows that a one-time reduction would save more lives. In addition, because nicotine levels in cigarettes can be readily reduced, there is also no logistical justification to take a step-down approach. In fact, setting multiple future

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[27] Dorothy K. Hatsukami et al., *Reduced nicotine content cigarettes: effects on toxicant exposure, dependence and cessation*, 105 ADDICTION 343 (2010), https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4565618/pdf/nihms-718685.pdf.

[28] *Id*; Dorothy Hatsukami, Address at the Society for Research on Nicotine and Tobacco Presidential Symposium, Reducing Nicotine Content in Cigarettes: A Discussion of the Evidence and Policy Implications (Feb. 21, 2018).

[29] Sarah S. Dermody et al., *Greater reductions in nicotine exposure while smoking very low nicotine content cigarettes predict smoking cessation*, 24 TOBACCO CONTROL 536 (2015), https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4784094/pdf/nihms-735134.pdf.

targets for nicotine levels would arguably be more burdensome for the industry than a single lower target that will remain steady for the indefinite future. A single target for nicotine levels is also more efficient for the FDA to enforce, requiring consistent inspections at one particular level rather than multiple levels over a period of years. In order to maximize the public health benefits of the rule, the agency must mandate one single target for nicotine levels.

Whether the proposed standard is characterized as "minimally-addictive" or "non-addictive" is less important than the FDA establishing a nicotine level that is low enough to maximize the public health benefits of a proposed rule. Because the risk of death and disease for combustible tobacco products is so high and the FDA's authority is so comprehensive, there is no reason not to mandate a nicotine level that does not create or sustain addiction. A sufficiently low level will allow smokers to quit entirely rather than just reducing their consumption.[30] This will also mean that even if youth experiment with combustible products, the products will not cause addiction, preventing the gateway effect, in which youth become addicted to nicotine through non-combustible products like e-cigarettes and then, to maintain their addiction, transition to combustible products which have been engineered to give the most effective dose of nicotine. The gateway effect is demonstrated by PATH research showing that in a group of youth participants, having ever used e-cigarettes was significantly related to progression to future, established cigarette smoking, past 30-day cigarette use, and to current established smoking.[31] At least one other study supports this finding, wherein youth respondents who were ever e-cigarette users and increased e-cigarette use over time were statistically more likely to initiate smoking, and a direct effect was found between ever using an e-cigarette and smoking initiation.[32]

c. A Product Standard for Nicotine in Non-Combustible Products Should be a Step-Down Reduction.

In order to finally break the cycle of nicotine addiction, the FDA must reduce nicotine to non-addictive levels in all tobacco products. While there is no question that the most significant portion of tobacco-caused disease and death results from the use of combustible products, non-combustible products are not without harm. An immediate reduction in nicotine for combustible products will cause a massive exodus from those products, ideally with a large portion of smokers quitting

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[30] Dorothy K. Hatsukami et al., *Dose-Response Effects of Spectrum Research Cigarettes*, 15 NICOTINE & TOBACCO RES. 6, 1113 (2013).
[31] Benjamin W. Chaffee et al., *Electronic Cigarette Use and Progression from Experimentation to Established Smoking*, 141 PEDIATRICS 1 (2018), https://www.ncbi.nlm.nih.gov/pubmed/29507167.
[32] Katherine East et al., *The Association Between Smoking and Electronic Cigarette Use in a Cohort of Young People,* 62 J. ADOLESCENT HEALTH 539 (2018), https://www.ncbi.nlm.nih.gov/pubmed/29499983.

entirely. There will also be smokers who move to non-combustible products like e-cigarettes, moist snuff, and snus.

It is important to remember that nicotine is not a benign substance. Nicotine is acutely toxic; nicotine increases the risk of tobacco-attributable disease by activating multiple biological pathways; nicotine exposure during fetal development and during adolescence has lasting adverse consequences for brain development; and nicotine contributes to multiple adverse outcomes for maternal and fetal health during pregnancy. In addition, all non-combustible tobacco products carry some risk, as e-cigarette aerosol and smokeless tobacco contain numerous toxicants that are capable of causing unique health harms such as organ damage and disease.[33]

Nicotine exposure can also result in various epigenetic modifications in both animals and humans, and these modifications can be passed down to offspring if they occur in the parents' sperm or eggs. These epigenetic markers are capable of inducing negative health and behavioral outcomes such as cognitive defects, hyperactivity, asthma, and susceptibility to drug abuse.[34] In fact, animal models have demonstrated that prenatal nicotine exposure can result in asthma not just in parents' children, but also in the grandchildren and even the great-grandchildren.[35] This can occur not only from maternal exposure to nicotine, but also through paternal exposure prior to conception.[36] In addition to the personal harms enacted by nicotine on an individual, nicotine also has the potential to harm future generations.

Gradually reducing the nicotine content in non-combustible products mimics a therapeutic approach to nicotine cessation, wherein a user steps down her or his nicotine level over time using nicotine replacement therapies. This more gradual transition for non-combustible products allows smokers to switch to non-combustible products that can still satisfy their nicotine cravings but will slowly

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[33] Chad A. Lerner et al., *Vapors Produced by Electronic Cigarettes and E-Juices with Flavorings Induce Toxicity, Oxidative Stress, and Inflammatory Response in Lung Epithelial Cells and in Mouse Lung*, 10 PLOS ONE 1 (2015), https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4319729/pdf/pone.0116732.pdf; Deeksha Bhartiya et al., *In-silico study of toxicokinetics and disease association of chemicals present in smokeless tobacco products*, 95 REG. TOXICOLOGY AND PHARMACOLOGY 8 (2018), https://www.ncbi.nlm.nih.gov/pubmed/29505798.

[34] Nicole L. Yohn et al., *Multigenerational and Transgenerational Inheritance of Drug Exposure: The Effects of Alcohol, Opiates, Cocaine, Marijuana, and Nicotine,* 118 PROGRESS IN BIOPHYSICS AND MOLECULAR BIOLOGY 21 (2015); Frances M. Leslie, *Multigenerational Epigenetic Effects of Nicotine on Lung Function*, 11 BMC MEDICINE 27 (2013).

[35] Virender K. Rehan et al., *Perinatal Nicotine Exposure Induces Asthma in Second Generation Offspring,* 10 BMC MEDICINE 129 (2012); Virender K. Rehan et al., *Perinatal Nicotine-Induced Transgenerational Asthma,* 305 AM. J. OF PHYSIOLOGY-LUNG CELLULAR AND MOLECULAR PHYSIOLOGY L501 (2013).

[36] Jingbo Dai et al., *Paternal Nicotine Exposure Defines Different Behavior in Subsequent Generation via Hyper-Methylation of mmu-miR-15b,* 7 SCI. REP. 7286 (2017).

transition them off of tobacco products entirely. Such a policy would eventually leave only therapeutic nicotine products, which are the safest method of nicotine delivery, with a level of nicotine that reduces withdrawal symptoms and supports cessation. The agency could also choose to make distinctions across categories of non-combustible products, reducing nicotine in some faster than others, based on relative risk. We suggest that the FDA ultimately allow only a five-year period between the initial reduction in nicotine in combustible products and the final step-down reduction in nicotine for non-combustible products, rendering them non-addictive. The longer that non-combustible products remain addictive, the more public health harm the products will cause.

II. The FDA Must Take Additional Steps to Maximize the Public Health Benefits of a Nicotine Product Standard.

While it is known that public health best practices couple policy change with a supportive infrastructure to facilitate compliance with new policies, in practice, too many times, a policy is enacted without accompanying supportive measures. Research demonstrates that, when placed in an experimental group receiving very low nicotine cigarettes, many smokers will either quit smoking altogether or switch to another product to maintain addiction.[37] In order to maximize quitting, the FDA should dedicate resources to educating smokers about the standard and the availability of cessation benefits. The agency must also increase access to cessation services and support new and innovative nicotine replacement therapies. Because of the potential for smokers to switch to non-combustible products as a result of a nicotine standard, the FDA should also simultaneously propose product standards for non-combustible products to maximize the benefit to public health.

A. The FDA Must Establish an Educational Campaign to Inform the Public About the Standard.

To achieve the greatest public health benefits of a nicotine product standard, the FDA must educate the public about the remaining risks of low nicotine tobacco products to avoid misperceptions of harm. Smokers and those who experiment with combustible products may misunderstand the rule as one that makes combustible products less harmful. Research participants have perceived the overall health risks as well as the risk of individual tobacco-caused diseases as significantly lower for very low nicotine cigarettes compared to average nicotine cigarettes.[38] In reality,

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[37] Dorothy Hatsukami, Address at the Society for Research on Nicotine and Tobacco Presidential Symposium, Reducing Nicotine Content in Cigarettes: A Discussion of the Evidence and Policy Implications (Feb. 21, 2018).

[38] Rachel L Denlinger-Apte et al., *Low Nicotine Content Descriptors Reduce Perceived Health Risks and Positive Cigarette Ratings in Participants Using Very Low Nicotine Content Cigarettes,* 19 NICOTINE & TOBACCO RES. 1149 (2017), https://www.ncbi.nlm.nih.gov/pubmed/28003507.

combustible products will be just as deadly when non-addictive. A federal education campaign will ensure that smokers understand that combustible products will be just as dangerous. Such a campaign would be most effective if it identified resources to help smokers quit tobacco products entirely.

Educational materials about the remaining harm of low nicotine tobacco products should address the fact that racial and ethnic groups can differ in susceptibility to nicotine addiction due to factors such as genetic variation in nicotine metabolism and clearance. For example, the Southwestern American Indian Tribal population has a higher prevalence of genotypes that result in reduced expression or activity of cytochrome P450 2A6 (CYP2A6), the primary enzyme that metabolizes nicotine, than the Northern Plains Tribal population.[39] Thus the Southwestern Tribal population overall has reduced nicotine metabolism, helping explain at least in part why they have less nicotine addiction, reduced smoking prevalence, and lower lung cancer mortality rates than the Northern Plains population. On the other hand, African American smokers tend to be more dependent on nicotine and have a higher risk of lung cancer despite generally smoking fewer cigarettes per day than white smokers in the U.S.[40] As such, a reduction in tobacco product nicotine levels may affect racial and ethnic groups differently. Because of this potential for differential impacts, the FDA should consider how it will tailor communications about this rule to groups that it will affect differently.

B. The FDA Must Increase Access to Cessation Services and Encourage Innovation in Nicotine Replacement Therapies.

Those who are most successful at quitting all combustible products do so with the aid of cessation services and the use of nicotine replacement therapies (NRTs), rather than simply switching to another product.[41] While the FDA does not provide cessation services directly, coordination with other agencies could ensure that when a final rule is implemented, current combustible users have access to quality, evidence-based cessation support. Additionally, the FDA should encourage innovation of NRTs to increase quit rates. The combination of NRT with the smoking

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[39] Julie-Anne Tanner et al., *Variation in CYP2A6 and nicotine metabolism among two American Indian tribal groups differing in smoking patterns and risk for tobacco-related cancer*, 27 PHARMACOGENETICS AND GENOMICS 169 (2017), https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5382092/pdf/nihms844044.pdf.

[40] Neal L. Benowitz et al., *Disposition Kinetics and Metabolism of Nicotine and Cotinine in African American smokers: Impact of CYP2A6 Genetic Variation and Enzymatic Activity*, 26 PHARMACOGENETICS AND GENOMICS 340 (2016), https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4892970/pdf/nihms766442.pdf.

[41] Dorothy Hatsukami, Address at the Society for Research on Nicotine and Tobacco Presidential Symposium, Reducing Nicotine Content in Cigarettes: A Discussion of the Evidence and Policy Implications (Feb. 21, 2018).

of a very low nicotine cigarette helps to address the increase in withdrawal symptoms reported by some very low nicotine cigarette study participants.[42]

C. The FDA Should Establish Product Standards for E-Cigarettes and Other Non-Combustible Products.

Given the potential for product switching, the FDA should take steps to address the health risks posed by the use of non-combustible tobacco products. To eliminate the use of dangerous commercial tobacco products, as discussed above, the FDA should promulgate a rule that would reduce nicotine in a gradual manner for all non-combustible tobacco products. Second, the FDA must simultaneously promulgate a rule that addresses flavors in all commercial tobacco products. The FDA should also establish other, complimentary product standards for non-combustible products, especially e-cigarettes. Because there are so many brands and varieties of e-cigarettes currently on the market, there have been many consumer safety concerns. For example, the FDA should consider promulgating a rule that addresses battery safety to address injuries caused by explosions. The FDA must also finalize its proposed rule to establish a standard for NNN levels in smokeless tobacco products.

The FDA must also address the threat that is posed by compact, concealable e-cigarettes that are particularly attractive to youth and have very high levels of nicotine. Because of the high nicotine output of Juul and other similar products, they pose particular addiction and health risks and are not well-suited to encourage complete cessation of nicotine. The FDA could address these products by setting a nicotine output threshold level for e-cigarette products at the same time that the agency establishes a maximum threshold for combustible products. Because the nicotine level that the user actually consumes is a complex combination of voltage and nicotine level in the nicotine solution, the FDA should focus this rule on the output of nicotine that the user consumes rather than the level of nicotine in the product.

III. The FDA Has Robust Authority to Mitigate Any Potential Countervailing Effects of a Nicotine Standard.

In its notice, the FDA has raised several potential complications that could diminish the public health benefits of a nicotine product standard. Foremost among those concerns is the possibility of the creation of illicit markets. In the U.S., interstate transportation and resale of tobacco products without proper tax assessment makes up the vast majority of illicit trade in tobacco products.[43] Because state borders

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[42] *Id.*

[43] *Understanding the U.S. Illicit Tobacco Market: Characteristics, Policy Context, and Lessons from International Experiences*, (Peter Reuter & Malay Majmundar, eds., 2015), https://www.nap.edu/read/19016/chapter/1.

within the U.S. are entirely open and there is significant tax disparity between states, this type of illicit trade is difficult to assess and police. The U.S. market for cigarettes is largely insular, that is, very few cigarettes are imported into the U.S. because domestic products make up the majority of the market.[44] Because a nicotine standard is imposed on the manufacturing of cigarettes, interstate transportation of domestically manufactured cigarettes would not circumvent a regulation. Illicit trade aimed at evading a nicotine standard would involve either smuggling cigarettes manufactured outside of the U.S., smuggling cigarettes into the U.S. market that were manufactured domestically but intended for exportation to global markets, or it would require cigarette manufacturers to intentionally manufacture non-compliant cigarettes and introduce them to the U.S. market.

Cross-border smuggling of cigarettes is not likely to have a large impact on a nicotine standard. In 2017, over 239 billion domestically manufactured cigarettes entered interstate commerce in the U.S., while only 7.8 billion cigarettes were imported.[45] Even if cigarette smugglers were able to bring in a number of cigarettes equal to the number ordinarily imported through legal channels, a feat that is likely impossible, they could only meet less than 4% of the current demand for cigarettes. Illicit cigarette smuggling will likely exist but it will not have a meaningful impact on the tobacco product market. It is also important to recognize that most smokers will not seek out illicit sources of cigarettes.[46] In addition, for a new cross-border smuggling illicit market to be established in the U.S., there must be individuals or enterprises willing to smuggle cigarettes into the country. Because of the variety of illicit products, primarily illegal drugs, that are already smuggled into the country with a much higher profit margin, there is little incentive to establish an illicit trade in cigarettes. The monetary rewards of smuggling illegal drugs are significantly higher. Domestic manufacturing facilities represent the only other possible source of illicit cigarettes. Rather than smuggling illicit cigarettes into the country, it is possible that existing cigarette factories could continue to manufacture cigarettes that do not comply with the nicotine standard and introduce them to the market through illicit channels. This could involve illegally diverting cigarettes that were manufactured for export or deliberately manufacturing illicit cigarettes. U.S. cigarette companies only exported about 5.2 billion of the 245 billion cigarettes manufactured in 2017. Even if all of these cigarettes were illegally diverted, this would be an insignificant source of illicit cigarettes. The illegal manufacturing and introduction of non-compliant cigarettes by large cigarette manufacturers is the most likely cause of an illicit market.

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[44] U.S. DEP'T. OF TREAS., *Statistical Report – Tobacco,* Dec. 2017, https://www.ttb.gov/statistics/2017/201712tobacco.pdf.
[45] *Id.*
[46] *Understanding the U.S. Illicit Tobacco Market: Characteristics, Policy Context, and Lessons from International Experiences*, (Peter Reuter & Malay Majmundar, eds., 2015), https://www.nap.edu/read/19016/chapter/1.

To combat cross-border smuggling, the FDA should coordinate with Customs and Border Patrol to increase enforcement of illegal cigarette smuggling after the implementation of a nicotine standard. The U.S. already has a robust infrastructure to prevent the illegal importation of illicit substances. In order to prevent legitimate cigarette manufacturers from manufacturing non-compliant cigarettes, the FDA must conduct unannounced inspections of domestic cigarette manufacturing facilities after the implementation of a nicotine standard, including comprehensive testing of products to ensure compliance. The FDA should also implement a track and trace system to better understand the inner-workings of the cigarette market. Such a system, which is recommended by the World Health Organization and has already been successfully implemented in other countries, could identify anomalies that would alert the agency to illicit behavior.[47] The FDA should not, however, wait until a track and trace system is in place before moving forward with a product standard for nicotine.

The FDA must also establish a system whereby inspections related to compliance with a nicotine standard are conducted by the FDA and not by the tobacco industry. If the agency depends on tobacco industry self-certification of compliance with the nicotine standard, it is likely that the industry will not comply with the standard.[48]

In its notice, the FDA also raises the issue of smokers using e-cigarette liquid to adulterate cigarettes by increasing the nicotine content. A step-down nicotine standard for non-combustible products would mitigate this concern. While some people may attempt to evade the standard, this would become significantly more difficult and eventually impossible as nicotine is reduced to non-addictive levels in non-combustible products.

The FDA should also anticipate industry behavior that may reduce the benefits of a final rule. As has been mentioned throughout this comment, the commercial tobacco product market is dominated by a small handful of companies that have a long history of deceptive behavior.[49] Furthermore, the business model for the commercial tobacco product market depends on addiction. Given this truth, the FDA must anticipate that the tobacco industry will do whatever it can to undermine a nicotine standard by attempting to keep users addicted. Accordingly, the FDA should prohibit the sale of any product that is intended to be used or may be used to circumvent a nicotine standard by increasing nicotine in a reduced nicotine

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[47] Centers for Disease Control and Prevention, *Use of Tobacco Tax Stamps to Prevent and Reduce Illicit Tobacco Trade — United States, 2014,* 64 MORBIDITY AND MORTALITY WKLY. REP. 547 (2015), https://www.cdc.gov/mmwr/pdf/wk/mm6420.pdf.

[48] U.S. v. Philip Morris, Inc., 449 F. Supp. 2d 1 (D.D.C. 2006), *aff'd in relevant part*, 566 F.2d 1095 (D.C. Cir. 2009), *cert. denied*, 130 S.Ct. 3501 (2010).

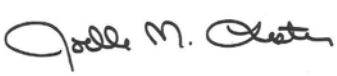
[49] *Id.*

cigarette. Finally, the FDA should ensure that a final rule will address not only nicotine, but any other nicotine analogues or additives that could be added to a cigarette to increase the addictiveness of the product while technically complying with a nicotine standard.

IV. Conclusion

We applaud the FDA for announcing this bold plan. The reduction of nicotine in tobacco products represents the potential end of the tobacco epidemic in the U.S. In order to ensure that the promise of this measure is fulfilled, the FDA must take a comprehensive approach to the rule. We urge the FDA to move forward as quickly as is possible.

Respectfully,



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Nonlinearity in Biology, Toxicology, and Medicine: Vol. 1, No. 2, pp. 179-198 (2003)

An Analysis of the Role of Tobacco-Specific Nitrosamines in the Carcinogenicity of Tobacco Smoke

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ABSTRACT

Cigarette smoke is a complex mixture consisting of more than 4500 chemicals, including several tobacco-specific nitrosamines (TSNA). TSNA typically form in tobacco during the post-harvest period, with some fraction being transferred into mainstream smoke when a cigarette is burned during use. The most studied of the TSNA is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK has been shown to be carcinogenic in laboratory animals. Studies examining the carcinogenicity of NNK frequently are conducted by injecting rodents with a single dose of 2.5 to 10 μmol of pure NNK; the amount of NNK contained in all of the mainstream smoke from about 3700 to 14,800 typical U.S. cigarettes. Extrapolated to a 70-kg smoker, the carcinogenic dose of pure NNK administered to rodents would be equivalent to the amount of NNK in all of the mainstream smoke of 22 to 87 million typical U.S. cigarettes. Furthermore, extrapolating results from rodent studies based on a single injection of pure NNK to establish a causative role for NNK in the carcinogenicity of chronic tobacco smoke exposure in humans is not consistent with basic pharmacological and toxicological principles. For example, such an approach fails to consider the effect of other smoke constituents upon the toxicity of NNK. *In vitro* studies demonstrate that nicotine, cotinine, and aqueous cigarette "tar" extract (ACTE) all inhibit the mutagenic activity of NNK. *In vivo* studies reveal that the formation of pulmonary DNA adducts in mice injected with NNK is inhibited by the administration of cotinine and mainstream cigarette smoke. Cigarette smoke has been shown to modulate the metabolism of NNK, providing a mechanism for the inhibitory effects of cigarette smoke and cigarette smoke constituents on NNK-induced tumorigenesis. NNK-related pulmonary DNA adducts have not been detected in rodents exposed to cigarette smoke, nor has the toxicity of tobacco smoke or tobacco smoke condensate containing marked reductions in TSNA concentra-

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tions been shown to be reduced in any biological assay. In summary, there is no experimental evidence to suggest that reduction of TSNA will reduce the mutagenic, cytotoxic, or carcinogenic potential of tobacco smoke.

Key Words: mainstream cigarette smoke, tobacco-specific nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

NNK AND OTHER TSNA IN TOBACCO AND TOBACCO SMOKE

Nitrosamines have been reported to be constituents of food, beverages, air, cosmetics, and industrial environments; accordingly, these chemicals have been an intensive topic of research and review for many years (IARC 17 1978; Banbury Report 1982; Loeppky and Michejda 1984; Magee 1996; Tricker 1997; Lin 1990; Preussmann and Eisenbrand 1984; Preston-Martin and Correa 1989; Magee 1989; Tricker *et al.* 1989; Startin 1996; Eisenbrand *et al.* 1996; Scanlan 1999;). It is commonly accepted that humans are exposed to nitrosamines on a daily basis; however, the precise levels of exposure and the significance of such exposure remains inconclusive (Tricker 1997).

Tobacco consumption represents an additional source of nitrosamine exposure. Tobacco-specific nitrosamines (TSNA) are a class of nitrosamines believed to occur only in tobacco, and have been reported as being present in a wide variety of tobacco-related products (Hoffmann *et al.* 1980; Adams *et al.* 1984; IARC 38 1985; Surgeon General's Report 1989; Hoffmann *et al.* 1991; Tricker *et al.* 1991; Hoffmann *et al.* 1994; Hoffmann and Hoffmann 1997; Hoffmann *et al.* 1997; Hoffmann and Hoffmann 1998; Hecht 1999). Known TSNA include 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [NNK], 4-(methylnitrosamino)-4-(3-pyridyl) butanal [NNA], *N'*-nitrosonornicotine [NNN], *N'*-nitrosoanabasine [NAB], *N'*-nitrosoanatabine [NAT], 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol [NNAL], 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol [*iso*-NNAL], and 4-(methylnitrosamino)-4-(3-pyridyl) butanoic acid [*iso*-NNAC] (Hecht and Tricker 1999). *iso*-NNAL and *iso*-NNAC rarely occur in mainstream cigarette smoke (Hecht and Tricker 1999). NNK, NNN, and NNAL are mutagenic *in vitro* and carcinogenic when administered to laboratory rodents (Boyland *et al.* 1964; Hoffmann *et al.* 1984). NAT and NAB demonstrate little or no mutagenic potential during *in vitro* testing nor carcinogenic activity in laboratory animals (Hoffmann et al. 1984; Padma et al. 1989).

Green and freshly harvested tobaccos are virtually free of TSNA (Green and Rodgman 1996; Caldwell and Conner 1990; Parsons *et al.* 1986; Spiegelhalder and Bartsch 1996; Brunnemann *et al.* 1982; Fischer *et al.* 1990). It is recognized that TSNA form during the post-harvest processing (e.g., curing) to which tobacco is subjected (Andersen *et al.* 1989; Djordjevic *et al.* 1989). Significant efforts have been expended toward studying the mechanism by which TSNA are formed (Peele 1995). TSNA are recognized as being formed when tobacco alkaloids (e.g., nicotine and nornicotine) are nitrosated (Tricker and Preussmann 1988). It has been postulated that, in the case of air curing of Burley tobacco, TSNA form as a result of microbial-

mediated conversion of nitrate to nitrite, coupled with the subsequent reaction of nitrate-derived chemical species with alkaloids present in the tobacco (Peele *et al.* 1995; Chamberlain and Chortyk 1992; Hecht 1998; Hamilton *et al.* 1982; Burton *et al.* 1992; Bush *et al.* 1995; Wiernik *et al.* 1995). In addition, there have been studies examining the potential impact of factors such as temperature of curing barns, humidity, amount of nitrogenous fertilizer used in growing, and amount of shade vs. sunlight on TSNA formation (Tso 1990; Davis and Nielsen 1999). More recently, the presence of NO_x gases produced from heating units used during flue-curing processes of Virginia tobacco has been shown to be a contributing factor to TSNA formation (Peele *et al.* 1999).

Typically, TSNA are not formed from tobacco pyrolysis; rather some fraction of the TSNA formed within tobacco during its curing process is transferred in mainstream tobacco smoke as preformed TSNA (Fisher *et al.* 1990). This is supported by the fact that the smoke generated by cigarettes made from low TSNA tobacco delivers low yields of TSNA in mainstream smoke (Peele *et al.* 1995; Doolittle *et al.* 2001). The amount of TSNA reported to be present in tobacco smoke varies among publications; probably due in part to differences in agricultural variations inherent in different crop years, tobacco curing techniques, the designs of the tested cigarette, the blends of tobacco used in cigarette manufacture, and smoking conditions. Furthermore, the various analytical methods employed to measure TSNA levels may contribute to this observed variability, leading some investigators to point out that earlier values reported in the scientific literature may be exaggerated due to artifact formation inherent in the earlier methodologies (Green and Rodgman 1996; Caldwell and Conner 1990).

Recent investigations have focused on the amount of TSNA, especially NNK, that a smoker is exposed to during smoking. Djordjevic *et al.* (2000) examined observed delivery of NNK and compared these data with delivery calculated using Federal Trade Commission (FTC) data. The actual observed delivery was nearly two times higher than delivery calculated using FTC data. Using observed delivery values (Djordjevic *et al.* 2000), a smoker of low-yield nicotine cigarettes ($\leq$ 0.8 mg/cigarette) would be exposed to approximately 187 ng NNK/cigarette, while a smoker of medium-yield nicotine cigarettes (0.9 to 1.2 mg/cigarette) would be exposed to approximately 251 ng NNK/cigarette.

Some scientists have hypothesized that ingested tobacco alkaloids, such as nicotine, might contribute to TSNA formation within the human body (Hoffmann *et al.* 1994; Hecht and Hoffmann 1989). Others have published data that support the conclusion that endogenous TSNA formation does not occur (Fischer *et al.* 1990; Caldwell *et al.* 1991; Meger *et al.* 1995; Adlkofer 1995; Spiegelhalder and Fischer 1990; Hecht *et al.* 1999; Tricker *et al.* 1993). The hypothesis of endogenous TSNA formation conflicts with recent evidence that smoking cessation therapies that involve the administration of nicotine in the form of gum, patch, and/or inhalers do not lead to endogenous TSNA formation (Hecht *et al.* 1999).

GENOTOXICITY OF NNK

N-nitrosamines require metabolic activation by cytochromes P_{450} for the expression of genotoxicity. NNK metabolism by a P_{450}-mediated α-hydroxylation pathway leads to several intermediates, some of which are genotoxic (Figure 1) (Hecht and Tricker 1999; Atalla and Maser 1999; Ren *et al.* 1999; Hecht *et al.* 1997). Moreover, NNAL, a genotoxic product of NNK carbonyl reduction may undergo α-hydroxylation resulting in the formation of additional genotoxic metabolites (Hecht and Tricker 1999; Atalla and Maser 1999; Ren *et al.* 1999; Hecht *et al.* 1997). Detoxication pathways include glucuronidation of NNAL and pyridine-*N*-oxidation of both NNK and NNAL (Hecht and Tricker 1999; Atalla and Maser 1999; Ren *et al.* 1999; Hecht *et al.* 1997).

TSNA and cigarette smoke condensate are both mutagenic in the Ames assay in the presence of S9 metabolic activation (Palma *et al.* 1989; Lee *et al.* 1996). However, there is no evidence to suggest that the small amount of NNK in cigarette smoke contributes to the mutagenicity observed for cigarette smoke condensate. Approximately 200 μg of pure NNK is required to demonstrate mutagenicity in the Ames assay using strain TA1535, the most sensitive strain for base pair mutagens commonly associated with *N*-nitrosamines (Lee *et al.* 1996). The dose of NNK required to elicit a moderate mutagenic response (200 μg) is equivalent to the amount of NNK yielded by approximately 2985 Kentucky 1R4F reference cigarettes smoked under standard FTC smoking conditions (Borderding *et al.* 1997). Since the amount of cigarette smoke condensate present in approximately 0.01 1R4F cigarettes (100

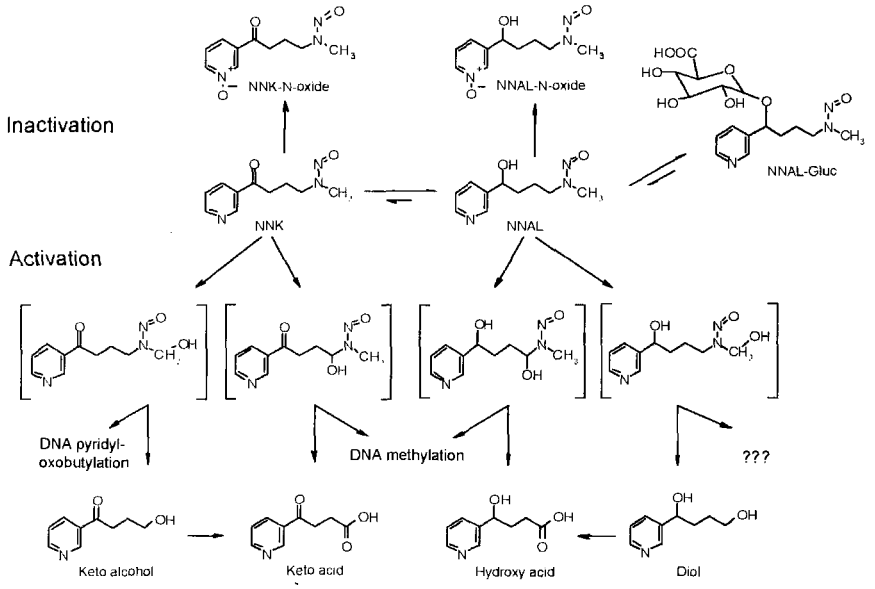

Figure 1. NNK metabolism pathways based on studies in laboratory animals. (From Hecht and Tricker, 1999.)

μg of CSC) is sufficient to demonstrate a substantial mutagenic response in the Ames test, it follows that the mutagenic response is not being driven by the level of TSNA in CSC. Furthermore, mainstream smoke from cigarettes generated using low TSNA tobacco failed to demonstrate reduced mutagenic potential within the Ames assay (Doolittle *et al.* 2001). Therefore, several lines of experimental evidence indicate that there are insufficient quantities of TSNA in tobacco smoke to contribute to the mutagenicity of tobacco smoke observed in the Ames test.

TUMORIGENICITY OF NNK IN LABORATORY ANIMALS

Tobacco-specific nitrosamines such as NNK are rodent carcinogens (Hecht and Tricker 1999; Hecht *et al.* 1978; Hecht *et al.* 1988; Belinsky *et al.* 1990; Hoffmann *et al.* 1993; Hecht *et al.* 1989; Hecht *et al.* 1986; Hecht *et al.* 1983) when administered in pure form. Hecht *et al.* (1989) has shown that NNK induces lung adenomas in A/J mice in a dose-response manner within 4 months of a single intraperitoneal (i.p.) injection. Tobacco-specific nitrosamines have also been shown to be carcinogenic as a consequence of oral cavity implantation and skin painting (Hecht *et al.* 1986). At present, there are no published data demonstrating TSNA to be carcinogenic via inhalation or respiratory tract exposure.

The fact that systemically administered TSNA produce lung adenomas in rodents have led some investigators to hypothesize that TSNA may be an important risk factor for lung cancer development in smokers (Hecht *et al.* 1989; Hecht *et al.* 1986; Hecht *et al.* 1983). However, extrapolation of the carcinogenic dose used in rodents results in unachievable "pack-year equivalents" per smoker (discussed in next section). Furthermore, a simplistic extrapolation of results obtained with pure TSNA to tobacco smoke is not supported by published animal studies demonstrating that tobacco extracts and/or smoke may actually reduce the carcinogenicity of pure TSNA in rodents (Hecht *et al.* 1986; Finch *et al.* 1996). In one such study, a solution of NNN and NNK solubilized in water and administered to male F344 rats via oral swab induced statistically significant ($p<0.05$) increases in oral cavity tumors as compared to vehicle control (Hecht *et al.* 1986). However, when the animals received the same dose of NNN and NNK along with snuff extract, there was a statistically significant decrease in oral cavity tumors whencompared with animals treated with pure NNN and NNK (Hecht *et al.* 1986).

A recent study has reported that whole-body inhalation exposure of A/J mice to 11% mainstream cigarette smoke and 89% sidestream cigarette smoke, used as an experimental surrogate for environmental tobacco smoke (ETS), resulted in a tumorigenic response provided that a 4 month post-exposure recovery period is incorporated into the experimental design (Witschi *et al.* 1997). A/J mice were similarly exposed to the same surrogate for whole ETS as well as HEPA-filtered ETS surrogate to remove the particulate phase of the smoke, so that the smoke consisted primarily of gas phase constituents (Witschi *et al.* 1997). Both exposures resulted in similar numbers of lung adenomas even though the concentration of NNK (mean

± SD) in the whole ETS surrogate and gas-phase ETS surrogate exposure atmospheres was 3.9 ± 3.5 and 0.29 ± 0.28 μg/m^3, respectively. Based on these values, the authors concluded that NNK was not the causative agent in the observed adenomas (Witschi *et al.* 1997). Finally, smoke from cigarettes made with low TSNA tobacco gave essentially the same biological response in a 90-day inhalation study in rats as the smoke from cigarettes made without reduced TSNA tobacco (Kinsler *et al.* 2002). Moreover, a comparative 30-week dermal study using SENCAR mice and comparing the cigarette smoke condensate (CSC) from low TSNA tobacco with the CSC from cigarettes made without reduced TSNA tobacco showed no statistically significant differences in numbers of dermal tumors (Hayes *et al.* 2003).

DOSE COMPARISONS OF NNK USED IN ANIMAL STUDIES VERSUS SMOKER EXPOSURE

TSNA have been found to be carcinogenic in the lungs of rats, mice, and hamsters when injected systemically (Hecht *et al.* 1978; Hecht *et al.* 1988; Belinsky *et al.* 1990; Hoffmann *et al.* 1993; Hecht *et al.* 1989; Hecht *et al.* 1986; Hecht *et al.* 1983). However, as shown in Table 1 extrapolation of the carcinogenic dose used in rodent studies results in unachievable "pack-year equivalents" per smoker. Actually, to mimic the mouse exposure data, a smoker would need to smoke all of the cigarettes at once since rodents receive a single injection of NNK. Also, one needs to also assume 100% absorption of NNK from the smoke. These dose calculations are based on an average NNK yield of market full flavor cigarettes smoked under FTC conditions, and serve to compare the dosimetry reported in systemically injected mice versus the dose in cigarettes that a smoker would have to consume. When considering the relevance to human smokers of the doses employed during animal studies, it is important to remember that over 40 years of smoking, a three pack-a-day smoker would smoke 876,000 cigarettes, or 43,800 packs. The 876,000 cigarettes would be approximately 2% to 8% of the 11 to 43 million cigarettes required to yield the dose of NNK reported to be carcinogenic in mice scaled to human body weight.

Some investigators have hypothesized a possible additive effect of individual TSNA in tobacco smoke. In the case of TSNA, there are 182 ng of NAT, 158 ng of NNN, and 135 ng of NNK per typical U.S. market full flavor cigarette (Chepiga *et al.* 2000); the total of all three TSNA would be about 475 ng per cigarette. Even assuming that NNN and NAT are as carcinogenic as NNK in rodents, which they are not (Hoffmann *et al.* 1984; Padma *et al.* 1989), one would still be considering unrealistic "pack-year equivalents" per smoker to yield the doses demonstrated to be carcinogenic in rodents (i.e., 10 to 40 packs per day for 40 years).

A recent study (Djordjevic *et al.* 2000) compared the amounts of NNK delivered to a smoker using the Federal Trade Commission (FTC) specified machine-smoking protocol (35-ml puff volume drawn for 2 s once per min) vs. data from actual smokers. Compared with the FTC protocol values, smokers of low-yield cigarettes

Table 1. Extrapolation of rodent bioassay results to human smokers.

Animal Model	A/J Mouse	
	Minimum Dose	Maximum Dose
Total NNK Dose (mg/25 gram mouse) required to induce significant incidence of lung tumors	0.52 mg	2.07 mg
Total NNK Dose (mg/kg body weight) required to induce significant incidence of lung tumors	20.8 mg	82.8 mg
NNK/Cigarette (Chepiga, 2000)	135 ng	
Total Cigarette Equivalence/kg Mouse Body Weight	~154,000	~613,000
Equivalent Dose of NNK in a 70 kg Smoker	1456 mg	5796 mg
Equivalent Number of Cigarettes	10,785,185	42,933,333
Comparison to Smoker (packs/day for 40 yrs)	37 packs	147 packs
Comparison to Smoker (Years of smoking 2 packs/day)	739 years	2941 years

(≤ 0.8 mg of nicotine per cigarette) and medium-yield cigarettes (0.9 to 1.2 mg of nicotine per cigarette) took statistically significantly larger puffs (48.6 and 44.1 ml, respectively) at statistically significantly shorter intervals (21.2 and 18.5 s, respectively), and drew larger total smoke volumes. Compared with the FTC yield for NNK per cigarette for Kentucky reference 1R4F, values of NNK per cigarette of smokers was approximately 2.5-fold higher for low-yield cigarettes and approximately 3.2-fold higher for medium-yield cigarettes. Using these values, a smoker would still need to smoke two packs per day for more than 300 years to be exposed to the low dose of NNK used in rodent studies and two packs per day for more than 1400 years for the equivalent of the higher dose used in rodent studies.

One of the more significant studies carried out to look at the dose-response relationship of the induction of pulmonary neoplasia in the Fischer 344 rat was by Belinsky et al. (1990). The lowest dose of NNK used to induce lung adenomas was 6 mg/kg, which is equivalent to over 44 thousand cigarettes/kg using 135 ng NKK/cigarette, as stated previously. The authors stated that this dose of NNK was similar to the dose of NNK that a smoker would be exposed to during a lifetime of smoking. This calculation apparently assumes that a smoker consumes 10 packs/day for 40 years.

INHIBITION OF THE BIOLOGICAL ACTIVITY OF NNK BY TOBACCO SMOKE AND SELECTED CONSTITUENTS

The tobacco-specific nitrosamine, NNK, requires metabolic activation to express its carcinogenic effects. However, there are competing detoxication pathways (Hecht 1994). The major metabolic pathway for NNK (in most tissues) involves conversion to NNAL via reduction of the NNK carbonyl group (Figure 1). This reaction occurs rapidly in rodents, primates, and human tissues (Smith *et al.* 1992; Castonguay *et al.* 1983). α-Hydroxylation of the methylene groups adjacent to the *N*-nitroso nitrogen of NNK and NNAL yields the corresponding keto acid and hydroxy acid, with liberation of the methylating agent, methanediazohydroxide. α-Hydroxylation of the methyl group in NNK ultimately yields the keto alcohol (also referred to as HPB), which can be oxidized to keto acid. The reactive intermediate, α-hydroxymethyl-NNK can decompose and react by pyridyloxobutylation of DNA and hemoglobin to form HPB-releasing adducts. α-Methyl hydroxylation of NNAL produces the major end product of 4-(3-pyridyl)butane-1,4,diol (diol), with no existing evidence to suggest that this metabolic pathway results in adduct formation (Richter *et al.* 2000). NNK and NNAL can be pyridine N-oxidized to form either the NNK or NNAL-N-oxide or can be conjugated to form NNAL-glucuronide, all of which are nongenotoxic metabolites that are readily excreted in urine.

Nicotine, cotinine, cigarette smoke, and aqueous cigarette "tar" extract (ACTE) have all been shown to inhibit the α-hydroxylation of NNK. Nicotine, as well as NNN and NAT demonstrate a dose-dependent inhibition of *in vitro* α-hydroxylation of NNK within rat oral tissue (Murphy and Heiblum 1990). Nicotine and cotinine both

reduce NNK metabolic activation by α-hydroxylation in the isolated and perfused rat liver, but not in the isolated and perfused rat lung (Schulze *et al.* 1998). Nicotine significantly reduces *in vivo* metabolic activation of NNK and excretion of α-hydroxylation metabolites (Richter and Tricker 1994), as well as significantly reduces [5-^{3}H]NNK binding of radioactivity (pyridyloxobutylation) to rat hemoglobin (Kutzer *et al.* 1994). Nicotine inhibits *in vitro* α-hydroxylation of NNK and protein binding (pyridyloxobutylation) in hamster lung explants (Schuller *et al.* 1991) and hepatic microsomal proteins (Castonguay and Rossignol 1992). Similar effects may occur *in vivo* since co-administration of nicotine results in a significant inhibition of NNK α-hydroxylation in the hamster (Richter *et al.* 2000). Compared to other rodent species, the hamster is relatively insensitive to NNK-induced lung tumorigenesis (Richter *et al.* 2000), most likely a consequence of limited NNK α-hydroxylation in the lung (Richter *et al.* 2000).

Lee *et al.* (1996) evaluated the mutagenicity of *N*-nitrosamines in the presence of nicotine and other structurally similar pyridine alkaloids. NNK, *N*-nitrosodimethylamine (NDMA), and NNN were tested in the Ames *Salmonella typhimurium* assay (in the presence of a metabolic activation system, S9) using strain TA1535, the most sensitive strain for base pair mutagens such as *N*-nitrosamines (Padma *et al.* 1989; Lee *et al.* 1996; Yahagi *et al.* 1977). Nicotine, cotinine, and aqueous cigarette "tar" extract (ACTE) all inhibited the mutagenicity of NDMA and NNK, while NNN mutagenicity was not affected. The induction of sister chromatid exchanges (SCE) in mammalian cells (CHO) by NNK in the presence of metabolic activation also was reduced significantly by nicotine and cotinine. Therefore, consistent with metabolism studies, nicotine and other tobacco constituents effectively inhibit the mutagenicity of NNK (Lee *et al.* 1996; Richter and Tricker 1994; Kutzer *et al.* 1994; Schuller *et al.* 1991).

While nicotine clearly inhibits the mutagenicity of NNK, other tobacco smoke constituents can also play a significant role. ACTE (aqueous cigarette tar extract), prepared from de-nicotinized cigarettes (containing significantly less nicotine [~0.08 mg/cig] than the Kentucky 1R4F reference cigarette [~0.9 mg/cig]) was tested for its effect on NNK mutagenicity (Lee *et al.* 1996). The inhibitory effects were almost identical suggesting that the inhibitory effect of ACTE on the mutagenicity of NNK is attributable to water-soluble constituents of cigarette smoke (Lee *et al.* 1996). The specific agent(s) in ACTE responsible for the inhibition of mutagenicity have not yet been identified.

NNAL is a potent pulmonary carcinogen in mice and rats (Hoffmann *et al.* 1993; Hecht *et al.* 1990) and is mutagenic in the Ames bacterial mutagenesis assay (Yahagi *et al.* 1977; Brown *et al.* 2001). Given the structural similarity between NNK and NNAL, and the metabolic activation of both by cytochromes P_{450}, we hypothesized that there may be a similar inhibition of NNAL metabolism, and consequently, inhibition of the mutagenic activity of NNAL by tobacco smoke and its pyridine alkaloid constituents. In a recent study, we evaluated the ability of two pyridine alkaloids (nicotine and cotinine), as well as ACTE to inhibit the mutagenicity of NNAL as assessed by *Salmonella typhimurium* strain TA1535 in the presence of a

metabolic activation system (S9) (Brown *et al.* 2001). Both pyridine alkaloids tested, as well as ACTE, inhibited the mutagenicity of NNAL in a concentration-dependent manner. These results demonstrate that tobacco smoke contains pyridine alkaloids, as well as other unidentified constituents that inhibit the mutagenicity of NNAL, a major metabolite of NNK (Brown *et al.* 2001). Due to the presence of these modulating agents in cigarette smoke, the biologically reactive dose of NNAL from cigarette smoking is likely to be much lower than predicted from studies comparing the biological activity of pure NNAL with plasma concentrations of NNAL.

A single intraperitoneal injection of NNK induces the formation of O^6-methylguanine in A/J mouse lung DNA (Brown *et al.* 1999; Peterson and Hecht 1991). O^6-MeG is a promutagenic base that induces guanine (G) to adenine (A) transition (Ronai *et al.* 1993). Any inhibition of the P_{450} -mediated α-hydroxylation reaction would be expected to reduce the formation of DNA-reactive species from TSNA, hence reducing genotoxic, mutagenic, and tumorigenic activities. Exposure of A/J mice to mainstream cigarette smoke (0, 400, 600, or 800 mg TSP/m^3) did not result in detectable levels of O^6-MeG in either lung or liver (Figure 2) (Brown *et al.* 1999). Moreover, A/J mice co-exposed to mainstream smoke (0, 400, 600, or 800 mg TSP/m^3) and a single i.p. administration of NNK (0, 3.75, or 7.5 μmol/mouse, sufficient to induce significant levels of O^6-MeG adducts) resulted in a significant dose-dependent reduction in NNK-induced lung and liver O^6-MeG (Figure 3) (Brown *et al.* 1999).

In a recent study designed to study metabolic inhibition/competition, A/J mice were exposed to mainstream cigarette smoke from the 1R4F cigarette (600 mg TSP/m^3) for 2 h, followed by a single i.p. Injection of NNK (7.5 μmol/mouse). Results from these studies demonstrated that tobacco smoke exposure significantly reduced NNK metabolic activation to the hydroxy acid and keto acid by 15% ($p=0.0029$) and 42% ($p<0.0001$), respectively, compared with sham-exposed (control) animals (Brown *et al.* 2001). Thus, co-administration of cigarette smoke reduces the metabolic activation of NNK (via α-hydroxylation) to DNA-reactive methylating species, a critical step in the induction of lung tumorigenesis in the A/J mouse.

Finally, phenethyl isothiocyanate (PEITC) is an effective inhibitor of lung tumorigenesis induced in rats and mice by the tobacco-specific carcinogen NNK (Hecht *et al.* 2000). However, studies have failed to demonstrate a protective effect for PEITC on tobacco smoke carcinogenesis in rodent models (Witschi *et al.* 1998; Witschi *et al.* 1999) providing additional evidence that NNK is not the causative agent in animal models of tobacco-smoke carcinogenesis.

HUMAN BIOMARKERS OF NNK METABOLISM

Although some have assumed that NNK metabolism is similar in laboratory rodents and in man, recent data do not support this assumption. Studies examining urinary metabolites of nicotine, NNK, NNN, and NNAL in rats (when compared with humans) revealed significant differences (Trushin and Hecht 1999; Hecht *et al.*

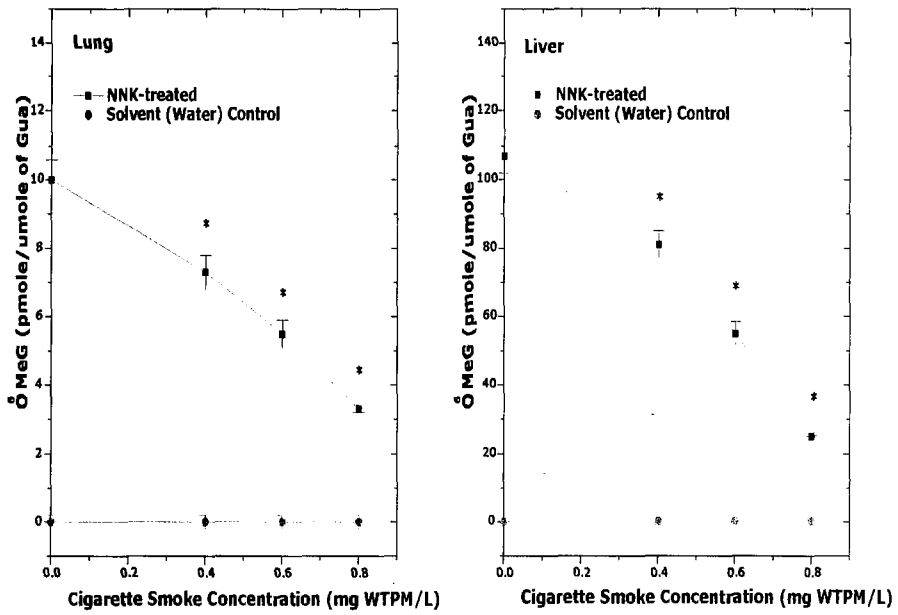

Figure 2. Dose-dependent reduction of O^6-MeG concentration by 1R4F cigarette smoke in A/J mice. Mice received a one-time, nose-only inhalation exposure of 1R4F cigarette smoke at 0, 0.4, 0.6, or 0.8 mg WTPM/L for 2 h to study the potential of cigarette smoke to inhibit NNK-induced O^6-MeG formation. The dosing of NNK (7.5 μmol/mouse, ip) was performed at the midpoint of the 2-h exposure. Mice were euthanized 4 h after NNK treatment and lung and liver DNA was analyzed for O^6-MeG by HPLC. (Mean ± SE; n = 18; * = $p < 0.05$). (From Brown *et al.* 1999.)

1999). An initial hypothesis of these studies was that urinary (S)-hydroxy acid could be a potential urinary biomarker of NNK and NNN α-hydroxylation in smokers. However, researchers discovered that the metabolism was significantly different between rodents and humans. For example, in the rat it is possible to distinguish the hydroxy acid derived from nicotine from that derived from TSNA (Trushin and Hecht 1999); this was not possible in humans (Hecht *et al.* 1999). Furthermore, when metabolism of NNK within precision-cut rodent and human liver and lung slices was compared, metabolism to NNAL was significantly higher in human tissues than in rodent tissues (Castonguay *et al.* 1983).

Incubation of NNK (3 to 10 μ*M*) with microsomes from human liver (Staretz *et al.* 1997) and lung (Smith *et al.* 1995) yields at least 95% NNAL, with little evidence of metabolism via α-hydroxylation, the predominant pathway in rodents (Hecht and Tricker 1999). Recent *in vitro* studies report that human buccal mucosa predominantly reduces NNK to NNAL (95 to 99%), in addition to metabolism via α-hydroxylation (0.6 to 3.8%) and pyridyl N-oxidation (0.3 to 2.2%) (Liu *et al.* 1993). In a study utilizing human lung slices (Castonguay *et al.* 1983), lung tissue

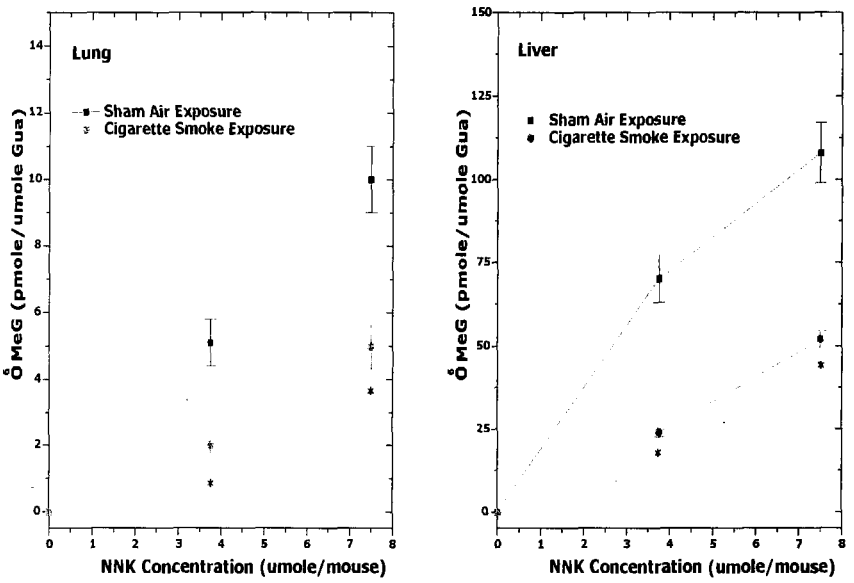

Figure 3. Effect of Kentucky reference 1R4F cigarette smoke (0.6 mg WTPM/L) on the lung and liver concentrations of O^6-MeG in NNK-treated A/J mice. Mice received a one-time, nose-only inhalation regimen of either HEPA-filtered and humidified air (control) or 1R4F cigarette smoke at the previously determined MNLD (0.6 mg WTPM/L) for 2 h to monitor the effect of cigarette smoke on the concentration of O^6-MeG in mice treated with NNK. A single ip dose of NNK (0, 3.75, or 7.5 μmol/mouse) was administered to mice at the midpoint of the 2-h exposure. Mice were euthanized 4 h after the NNK treatment, lung and liver DNA were analyzed for O^6-MeG by HPLC. (Mean ± SE; n = 18; * = $p < 0.05$). (From Brown *et al.* 1999.)

demonstrated a low capacity to metabolize NNK. Metabolism proceeded mainly via a low K_m (high affinity) reduction to NNAL (K_m 0.5 μ*M*; V_{max} 388 fmol/min/mg protein and K_m 39; V_{max} 21380), with a lower potential to form methylating and/or pyridyloxobutylating species. Consistent with this, 7-methyl-2-deoxyguanosine DNA adduct levels in the lungs of smokers (and nonsmokers) cannot be explained by differences in tobacco exposure, with pyridyloxobutylation undetectable in smokers' lungs (Blomeke *et al.* 1996). At a plausible level of NNK exposure, α-hydroxylation to the keto acid (K_m 690; V_{max} 13390) in the liver is unlikely due to the low K_m high-affinity reduction to NNAL (K_m 0.6; V_{max} 254 and K_m 44; V_{max} 11340) (Castonguay *et al.* 1983).

Hemoglobin (Hb) adducts from TSNA have been suggested as biomarkers of exposure for both tobacco smoke and smokeless tobacco. The metabolic activation of both NNK and NNN results in a common Hb adduct, releasing 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) after alkaline hydrolysis. Initial biomonitoring studies reported adduct levels significantly higher in the blood of smokers than in non-

smokers (Carmella *et al.* 1990; Falter *et al.* 1994; Atawodi *et al.* 1998), while Hb adduct levels were significantly higher for users of smokeless tobacco when compared with smokers in two of the studies (Carmella *et al.* 1990; Falter *et al.* 1994). Urinary excretion of the NNK metabolites, NNAL and NNAL-Gluc are about 120 times higher in smokers than in nonsmokers, but HPB-releasing hemoglobin adducts derived from NNK and NNN in both smokers and nonsmokers are frequently not much higher than assay background amounts (Hecht and Tricker 1999). In a study in which smokers and smokeless tobacco users demonstrated approximately the same level of NNK and NNN uptake, the mean adduct level was approximately 7 times higher in smokeless tobacco users than in smokers (Carmella *et al.* 1990; Falter *et al.* 1994). This leads to the conclusion that adduct levels in smokers may be lower than expected due to induction of metabolizing enzymes that detoxify TSNA in smokers (Falter *et al.* 1994; Atawodi *et al.* 1998) and/or that TSNA activation is inhibited by other smoke constituents (Lee *et al.* 1996; Brown *et al.* 2001; Brown *et al.* 1999; Brown *et al.* 2001).

CONCLUSION

Is it prudent to reduce NNK and other TSNA in tobacco products? Sure it is, to the extent possible. Reduced TSNA in tobacco products will result in reduced exposure to TSNA. Will such reduction mean a reduced cancer risk? That cannot be determined until smokers have used reduced TSNA products for several years.

A review of the scientific literature suggests the following: (1) NNK, a tobacco-specific nitrosamine, is found in cured tobacco and in tobacco smoke; (2) in pure form, NNK is toxic and mutagenic to cultured cells *in vitro*; (3) in pure form, NNK is carcinogenic in experimental animals; (4) extrapolated to man and based on the minimum amount of NNK required to cause tumors in A/J mice (the most sensitive rodent model), the amount of NNK found in the smoke from millions of cigarettes would be required to provide a carcinogenic equivalent to smokers; (5) the mutagenicity and carcinogenicity of NNK can be inhibited by nicotine and cotinine, as well as additional unidentified constituents of cigarette smoke; and (6) CSC or smoke from reduced TSNA cigarettes is similar in toxicity, mutagenicity, and carcinogenicity to CSC or smoke from cigarettes with current levels of TSNA.

Based on our review of the published literature, we conclude that there is neither direct nor convincing evidence that NNK or TSNA *in toto* play a significant role in the increased risk of lung cancer associated with cigarette smoking. Furthermore, there is no compelling experimental evidence that reducing the levels of TSNA in tobacco smoke will have a significant impact on the lung cancer risks associated with cigarette smoking.

REFERENCES

Adams, J.D., Lee, S.J., and Hoffman, D. (1984): Carcinogenic agents in cigarette smoke and the influence of nitrate on their formation. *Carcinogenesis* 5(2):221–223.

Adlkofer, F.X. (1995): Involvement of nicotine and its metabolites in the pathology of smoking-related diseases: Facts and hypotheses. In: *Effects of Nicotine on Biological Systems II,* edited by P.B.S. Clarke, M. Quik, F. Adlkofer, and K. Thurau. Birkhauser Verlag, Basel, Switzerland.

Andersen, R.A., Fleming, P.D., Burton, H.R., Hamilton-Kemp, T.R., and Sutton, T.G. (1989). *N'*-Acyl and *N'*-nitroso pyridine alkaloids in alkaloid lines of Burley tobacco during growth and air curing. *J Agric Food Chem* 37(1):44–50.

Atalla, A. and Maser, E. (1999). Carbonyl reduction of the tobacco-specific nitrosamine 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone (NNK) in cytosol of mouse liver and lung. *Toxicology* 139:155–166.

Atawodi, S.E., Lea, S., Nyberg, F., Mukeria, A., Constantinescu, V., Ahresn, W., Brueske-Hohlfeld, I., Fortes, C., Boffetta, P., and Friesen M.D. (1998). 4–Hydroxy-1– (3–pyridyl)-1–butanone-Hemoglobin adducts as biomarkers of exposure to tobacco smoke: Validation of a method to be used in multicenter studies. *Cancer Epidemiol Biomarkers Prev* 7:817–821.

Banbury Report 12: *Nitrosamines and Human Cancer,* edited by Peter N Magee, Cold Spring Harbor Laboratory (1982).

Belinsky, S.A., Foley, J.F., White, C.M., Anderson, M.W., and Maronpot, R.R. (1990): Dose-response relationship between O^6–methylguanine formation in Clara cells and induction of pulmonary neoplasia in the rat by 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone. *Cancer Res* 50:3772–3780.

Blomeke, B., Greenblatt, M.J., Van Doan, D. (1996). Distributions of 7–alkyl-2'-deoxyguanosine adduct levels in human lung. *Carcinogenesis* 17:741–748.

Borgerding, M.F., Bodnar, J.A., Chung, H.L., Mangan, P.P., Morrison, C.C., Risner, C.H., Rogers, J.C., Simmons, D.F., Uhrig, M.S., Wendelboe, F.N., Wingate, D.E., and Winkler, L.S. (1997). Chemical and biological studies of a new cigarette that primarily heats tobacco. Part 1. Chemical composition of mainstream smoke. *Food Chem Tox* 36:169–182.

Boyland, E., Roe, F. J.C., Gorrod, J.W., and Mitchley, B.C.V. (1964). The carcinogenicity of nitrosoanabasine, a possible constituent of tobacco smoke. *Br. J. Cancer* 18:265–270.

Brown, B.G., Chang, C-J. G., Ayres, P.H., Lee, C.K., and Doolittle, D.J. (1999). The effect of cotinine or cigarette smoke co-administration on the formation of O^6–methylguanine adducts in the lung and liver of A/J mice treated with 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone (NNK). *Tox Sci* 47:33–39.

Brown, B., Avalos, J., Lee, C., and Doolittle, D. (2001). The effect of tobacco smoke, nicotine, and cotinine on the mutagenicity of 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanol (NNAL). *Mut Res* 494 (1–2): 21–29.

Brown, B., Richter, E., Tricker, A., and Doolittle, D. (2001). The effect of a 2–h exposure to cigarette smoke on the metabolic activation of the tobacco-specific nitrosamine 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone (NNK) the A/J mice. *Chemico-Biological Interactions* 138:125–135.

Brunnemann, K.D., Hecht, S.S., and Hoffmann, D. (1982–3). *N*-nitrosamine: Environmental occurrence, in vivo formation and metabolism. *J Toxicol-Clin Toxicol* 19 (6 & 7): 661–688.

Burton, H.R., Naewanna, K.D., and Bush, L.P. (1992). Distribution of tobacco constituents in tobacco leaf tissue. I. Tobacco-specific nitrosamines, nitrate, nitrite, and alkaloids. *J Agric Food Chem* 40:1050–1055.

Bush, L.P., Cur, M, and Burton, H.R. (1995). Origin of nitrite-nitrogen for tobacco-specific N'-nitrosamine formation. *Coresta Information Bulletin,* Abstract 9814.

Caldwell, W.S. and Conner, J.M. (1990): Artifact formation during smoke trapping: An improved method for determination of *N*-nitrosamines in cigarette smoke. *J Assoc Off Anal Chem* 73(5): 783–789.

Caldwell, W.S., Greene, J.M., Plowchalk, D.R., and deBethizy, J.D. (1991): The nitrosation of nicotine: A kinetic study. *Chem Res Toxicol* 4:513–516.

Carmella, S.G., Kagan, S.S., Kagan, M., Foiles, P.G., Palladino, G., Quart, A.M., Quart, E., and Hecht, S.S. (1990). Mass spectrometric analysis of tobacco-specific nitrosamine hemoglobin adducts in snuff dippers, smokers, and nonsmokers. *Cancer Res* 50:5438–5445.

Castonguay, A., Lin, D., Stoner, G.D., Radok, P., and Furuya, K. (1983). Comparative carcinogenicity in A/J mice and metabolism by cultured mouse peripheral lung of *N'*-nitrosonornicotine, 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone and their analogues. *Cancer Res* 43:1223–1229.

Castonguay, A., Stoner, G.D., Schut, H.A.J., and Hecht, S.S. (1983). Metabolism of tobacco-specific *N*-nitrosamines by cultured human tissues. *Proc Natl Acad Sci USA* 80:6694–6697.

Castonguay, A. and Rossignol, G. (1992). Modulation of the activation of 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone by hamster lung microsomes to protein alkylating species. *Toxic in Vitro* 6:397–404.

Chamberlain, W.J. and Chortyk, O.T. (1992). Effect of curing and fertilization on nitrosamine formation in bright and Burley tobacco. *Beitr. Tobakforsch* 15:87–92.

Chepiga, T.A., Morton, M.J., Murphy, P.A., Avalos, J.T., Bombick, B.R., Doolittle, D.J., Borgerding, M.F., and Swauger, J.E. (2000). A comparison of the mainstream smoke chemistry and mutagenicity of a representative sample of the US cigarette market with two Kentucky reference cigarettes (K1R4F and K1R5F). *Food Chem Tox* 38:949–962.

Davis, D.L. and Nielsen, M.T. (1999): *Tobacco: Production, Chemistry and Technology.* World Agriculture Series, CORESTA, Blackwell Science Ltd., Oxford, England.

Djordjevic, M.V., Brunnemann, K.D., and Hoffmann, D. (1989). Identification and analysis of a nicotine-derived *N*-nitrosamino acid and other nitrosamino acids in tobacco. *Carcinogenesis* 10:1725–1731.

Djordjevic, M.V., Stellman, S.D., and Zang, E. (2000). Doses of nicotine and lung carcinogens delivered to cigarette smokers. *J Natl Cancer Inst* 92:106–111.

Doolittle, D.J., Avalos, J.T., Bombick, B.R., Putnam, K.P., Nestor, T.B., and Gentry, J.S. (2001). Biological studies on smoke condensates from cigarettes made with low nitrosamines flue-cured tobacco. *Tox Sci* 60:416.

Eisenbrand, G., Fuchs, A., and Koehl W. (1996): *N*-nitroso compounds in cosmetics, household commodities and cutting fluids. *Eur J Canc Prev* 5(1):41–46.

Falter, B., Kutzer, C., and Richter, E. (1994). Biomonitoring of hemoglobin adducts: aromatic amines and tobacco-specific nitrosamines. *Clin Investig* 72:364–371.

Finch, G.L., Nikula, K.J., Belinsky, S.A., Barr, E.B., Stoner, G.D., and Lechner, J.F. (1996). Failure of cigarette smoke to induce or promote lung cancer in the A/J mouse. *Cancer Letters* 99:161–167.

Fischer, S., Spiegelhalder, B., Eisenbarth, J., and Preussmann, R. (1990). Investigations on the origin of tobacco-specific nitrosamines in mainstream smoke of cigarettes. *Carcinogenesis* 11 (5):723–730.

Green, C.R. and Rodgman, A. (1996): The Tobacco Chemists' Research Conference: A half century forum for advances in analytical methodology of tobacco and its products. In: *Recent Advances in Tobacco Science, Volume 22: Fifty years of tobacco science and technology.*

Symposium proceedings of the 50th meeting, Tobacco Chemists' Research Conference, Richmond, VA, October 20–23, 1996.

Hamilton, J.L., Bush, L. and Lowe, R.H. (1982). Nitrate concentration changes during senescence and air curing of burley tobacco. *Tob Sci* 26:133–137.

Hayes, J.R., Stravanja, M.S., Meckley, D.R., Van Kampen, K.R., Nelson, P.R., Ayres, P.H., Mosberg, A.T., and Swauger, J.E. (2003). Comparative 30–week dermal tumor promotion study using SENCAR mice: comparison of cigarette smoke condensate from a reference cigarette containing direct-fire flue-cured tobacco and a test cigarette containing heat-exchange flue-cured tobacco. *The Toxicologist* 72 (S-1):p 86.

Hecht, S.S., Chen, C.B., Hirota, N., Ornaf, R.M., Tso, T.C., and Hoffmann, D. (1978): Tobacco-specific nitrosamines: Formation from nicotine *in vitro* and during tobacco curing and carcinogenicity in Strain A mice. *J Natl Cancer Inst* 60(4):819–824.

Hecht, S.S., Adams, J.D., Numoto, S., and Hoffmann, D. (1983). Induction of respiratory tract tumors in Syrian golden hamsters by a single dose of 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone (NNK) and the effect of smoke inhalation. *Carcinogenesis* 4:1287–1290.

Hecht, S.S., Rivenson, A., Braley, J., DiBello, J., Adams, J.D., and Hoffmann, D. (1986): Induction of oral cavity tumors in F344 rats by tobacco-specific nitrosamines and snuff. *Cancer Research* 46:4162–4166.

Hecht, S.S., Abbaspour, A., and Hoffmann, D. (1988): A study of tobacco carcinogenesis XLII. Bioassay in A/J mice of some structural analogues of tobacco-specific nitrosamines. *Cancer Letters* 42:141–145.

Hecht, S.S., Morse, M.A., Amin, S., Stoner, G.D., Jordan, K.G., Choi, C., and Chung, F.L. (1989). Rapid single-dose model for lung tumor-induction in A/J mice by 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone and the effect of diet. *Carcinogenesis* 10:1901–1904.

Hecht, S.S. and Hoffmann, D. (1989). The relevance of tobacco-specific nitrosamines to human cancer. *Cancer Surv* 8:271–294.

Hecht, S.S., Jordan, K.G., Choi, C-I., and Trushin, N. (1990). Effects of deuterium substitution on the tumorigenicity of 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone and 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanol in A/J mice. *Carcinogenesis* 11:1017–1020.

Hecht, S.S. (1994). Metabolic activation and detoxification of tobacco-specific nitrosamines—a model for cancer prevention strategies. *Drug Metab Rev* 26:373–390.

Hecht, S.S., Spratt, T.E., and Trushin, N. (1997). Absolute configuration of 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanol (NNAL) formed metabolically from 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone (NNK). *Carcinogenesis* 18:1851–1855.

Hecht, S.S. (1998). Biochemistry, biology, and carcinogenicity of tobacco-specific *N*-nitrosamines. *Chem Res Toxicol* 11(6): 559–603.

Hecht, S.S. (1999). Tobacco smoke carcinogens and lung cancer. *J Nat Canc Inst* 91(14): 1194–1210.

Hecht, S.S. and Tricker, A.R. (1999). *Analytical Determination of Nicotine and Related Compound and Their Metabolites*, Gorrod, J.W. and Jacob III, P., Eds., Elsevier, Amsterdam, The Netherlands.

Hecht, S.S., Carmella, S.G., Chen, M., Dor Koch, J.F., Miller, A.T., Murphy, S.E., Jensen, J.A., Zimmerman, C.L., and Hatsukami, D.K. (1999): Quantitation of urinary metabolites of a tobacco-specific lung carcinogen after smoking cessation. *Cancer Res* 59:590–596.

Hecht, S.S., Hatsukami, D.K., Bonilla, L.E., and Hochalter, J.B. (1999). Quantitation of 4–Oxo-4– (3–pyridyl) butanoic acid and enantiomers of 4–hydroxy-4– (3–pyridyl) bu-

tanoic acid in human urine: A substantial pathway of nicotine metabolism. *Chem Res Toxicol* 12:172–179.

Hecht, S.S., Kenney, P.M., Wang, M., Trushin, N., and Upadhyaya, P. (2000). Effects of phenethyl isothiocyanate and benzyl isothiocyanate, individually and in combination, on lung tumorigenesis induced in A/J mice by benzo [*a*] pyrene and 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone. *Cancer Lett* 150(1): 49–56.

Hoffmann, D., Chen, C-H.B., and Hecht, S. (1980): The role of volatile and nonvolatile *N*-nitrosamines in tobacco carcinogenesis. In: *Banbury Report 3: A Safe Cigarette?*, Gori, G.B. and Bock, F.g., Ed., pp. 113–127.

Hoffmann, D. Rivenson, A. Amin, S., and Hecht, S.S. (1984). Dose-response study of the carcinogenicity of tobacco-specific N-nitrosamine in F344 rats. *J Cancer Res Clin Oncol* 108:81–86.

Hoffmann, D., Hoffmann, L., and Wynder, E.L. (1991): Lung cancer and the changing cigarette. In: *Relevance to human cancer of N-nitroso compounds, tobacco and mycotoxins*, edited by I.K. O'Neill, J. Chen, and H. Bartsch, pp. 449–459. International Agency for Research on Cancer, IARC Scientific Publications No. 105, Lyon.

Hoffmann, D., Djordjevic, M.V., Rivenson, A., Zang, E., Desai, D., and Amin, S. (1993): A study of tobacco carcinogenesis. LI. Relative potencies of tobacco-specific *N*-nitrosamines as inducers of lung tumours in A/J mice. *Cancer Lett* 71:25–30.

Hoffmann, D., Brunnemann, K.D., Prokopczyk, B., Djordjevic, M.V. (1994): Tobacco-specific *N*-nitrosamines and *areca*-derived *N*-nitrosamines: Chemistry, biochemistry, carcinogenicity, and relevance to humans. *J Toxicol Environ Health* 41:1–52.

Hoffmann, D. and Hoffmann, I. (1997): The changing cigarette, 1950–1995. *J Toxicol Environ Health* 50:307–364.

Hoffmann, D., Djordjevic, M.V., and Hoffmann, I. (1997). The changing cigarette. *Prev Med* 26(4): 427–434.

Hoffmann, D. and Hoffmann, I. (1998): Tobacco Smoke Components (Letter to the Editor). *Beit zur Tabak Inter* 18(1):49–52.

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Some *N*-nitroso Compounds, Volume 17, May 1978.

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Tobacco smoking, Volume 38, 1985.

Kinsler, S., Ayres, P.H., and Mosberg, A.T. (2002). Rat subchronic inhalation study of smoke from cigarettes containing flue-cured tobacco cured either by direct-fired or heat exchanger curing processes. *The Toxicologist* 66:195.

Kutzer, C., Richter, E., Oehlmann, C., and Atawodi, S.E. (1994). Effects of nicotine and cotinine on NNK metabolism in rats. In: Clarke, B.P.S., Quick, M., Adlkofer, F., and Thurau, K., Eds. *Effects of Nicotine on Biological Systems II.* Birkhauser Verlag, Basel, Pp. 385–390.

Lee, C.K., Fulp, C., Bombick, B.R., and Doolittle, D.J. (1996): Inhibition of mutagenicity of *N*-nitrosamines by tobacco smoke and its constituents. *Mutat Res* 367:83–92.

Lin, J.K. (1990). Nitrosamines as potential environmental carcinogens in man. *Clin Biochem* 23(1):67–71.

Liu, Y.K., Sundqvist, Belinsky, S.A., Castonguay, A., Tjalve, H., and Grafstrom, R.C. (1993). Metabolism and macromolecular interaction of the tobacco-specific carcinogen 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone in cultured explants and epithelial cells of human buccal mucosa. *Carcinogenesis* 14:2383–2388.

Magee, P.N. (1989). The experimental basis for the role of nitroso compounds in human cancer. *Canc Surv* 8(2): 207–239.

Magee, P.N. (1996). Nitrosamines and human cancer: Introduction and overview. *Eur J Canc Prev* 5(1):7–10.

Meger, M., Tricker, A.R., Scherer, G., Adlkofer, F., and Klus, H. (1995): 4–(*N*-methylnitrosamino)-1–(3–pyridyl)-1–butanol and its *O*-glucuronide in smoker and nonsmoker urine. 1st Annual Scientific Conference of the Society for Research on Nicotine and Tobacco, San Diego Bay, CA, March 24–25, 1995.

Murphy, S.E. and Heiblum, R. (1990). Effect of nicotine and tobacco-specific nitrosamines on the metabolism of NNN and NNK by rat oral tissue. *Carcinogenesis* 11:11663–1666.

Nitrosamines and Related *N*-Nitroso Compounds-Chemistry and Biochemistry, edited by R.N. Loeppky and C.J. Michejda, American Chemistry Society Symposium Series 553 (1984).

Padma, P.R., Amonkar, A.J., and Bhide, S.V. (1989): Mutagenic and cytogenetic studies of *N*'-nitrosonornicotine and 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone. *Cancer Letters* 46:173–180.

Parsons, L.L., Smith, M.S., Hamilton, J.L., and MacKown, C.T. (1986). Nitrate reduction during curing and processing of burley tobacco. *Tob Sci* 30:100–103.

Peele, D.M., Danehower, D.A., and Goins, G.D. (1995). Chemical and biochemical changes during the flue-curing of tobacco. *Coresta Information Bulletin*, Abstract 9822.

Peele, D.M., Riddick, M.G., Edwards, M.E., Gentry, J.S., and Nestor, T.B. (1999). Formation of tobacco specific nitrosamines in flue-cured tobacco. CD-ROM version 11, Joint meeting of the CORESTA Agronomy & Phytopathology Study Groups. Suzhou, China, October 10–14, 1999.

Peterson, L.A., and Hecht, S.S. (1991). O^6–Methylguanine is a critical determinant of 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone tumorigenesis in A.J mouse lung. *Cancer Res* 51:5557–5564.

Preston-Martin, S. and Correa, P. (1989): Epidemiological evidence for the role of nitroso compounds in human cancer. *Canc Surv* 8(2):459–473.

Preussmann, R. and Eisenbrand, G. (1984). *N*-Nitroso carcinogens in the environment. In: *Clinical Carcinogens*, Vol. 2, Searle, C.E., Ed., pp. 829–868. American Chemical Society, ACS Monograph 182, Washington, D.C.

Ren, Q., Murphy, S.E., Danneneberg, A.J., Park, J.Y., Tephly, T.R., and Lazarus, P. (1999). Glucuronidation of the lung carcinogen 4–(methylnitrosamino)-1–(3–pyridyl)-1butanol (NNAL) by rat UDP-glucuronosyltransferase 2B1. *Drug Metab Dis* 27:1010–1016.

Richter, E. and Tricker, A.R. (1994). Nicotine inhibits the metabolic activation of the tobacco-specific nitrosamine 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone in rats. *Carcinogenesis* 15:1061–1064.

Richter, E., Friesenegger, S., Engl, J, and Tricker, A. R. (2000). Use of precision-cut tissue slices in organ culture to study metabolism of 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone (NNK) and 4–(methylnitosamino)-1–(3–pyridyl)-1–bunaol (NNAL) by hamster lung, liver, and kidney. *Toxicology* 144:83–91.

Ronai, Z., Gradia, S., Peterson, L.A., and Hecht, S.S. (1993). G to A transitions and G-T transversion in codon 12 of the K-ras oncogene isolated from mouse lung tumors induced by 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone (NNK) and related DNA methylating and pyridyloxobutylating agent. *Carcinogenesis* 714:2419–2422.

Scanlan, R.A. (1999): *N*-nitroso compounds in carcinogenesis: an update of human exposure. American Chemical Society, Abstracts of Papers — Part 1, 217th ACS National Meeting,

Anaheim, CA, March 21–25, 1999.

Schuller, H.M., Castonguay, A., Orloff, M., and Rossignol, G. (1991). Modulation of the uptake and metabolism of 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone by nicotine in hamster lung. *Cancer Res* 51:2009–2014.

Schulze, J., Schrader, E., Foth, H., Kahl, G.F., and Richter, E. (1998). Effect of nicotine or cotinine on metabolism of 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone (NNK) in isolated rat lung and liver. *Naunyn-Schmiedeberg's Arch Pharmacol* 357:344–350.

Smith, T.J., Guo, Z., and Gonzalez, F.J., (1992). Metabolism of 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone (NNK) by human lung and liver microsomes and cytochrome P450 expressed in hepatoma cells. *Cancer Res* 52:1757–1763.

Smith, T.J., Stoner, G.D., and Yang, C.S. (1995). Activation of 4–(methylnitrosamino)-1–(3–pyridyl)-1–butanone (NNK) in human lung microsomes by cytochrome P450, lipoxygenase, and hydroperoxides. *Cancer Res* 55:5566–5573.

Spiegelhalder, B. and Fischer, S. (1990): Formation of tobacco-specific nitrosamines. Abstract from the 15th International Cancer Congress, Hamburg, Germany, August 16–22, 1990. *J Cancer Res Clin Oncol* 116(2):1088.

Spiegelhalder, B. and Bartsch, H. (1996). Tobacco-specific nitrosamines. *Eur J Cancer Prev* 5(1):33–38.

Staretz, M.E., Murphy, S.E., and Patten, C.J. (1997). Comparative metabolism of the tobacco-related carcinogens, benzo [a] pyrene, 4(methylnitrosamino)-1–(3–pyridyl)-1–butanone, 4–(methylnitrosamino)-1–(3–pyridyl)-1–butano, and *N*'-nitrosonornicotine in human hepatic microsomes. *Drug Metab Dis* 25:154–162.

Startin, J.R. (1996). *N*-nitroso compounds in foods and drinks. *Eur J Canc Prev* 5(1): 39.

Tricker, A.R. and Preussmann, R. (1988). The occurrence of *N*-nitroso compounds [corrected] in zarda tobacco. *Canc Lett* 42:113–118.

Tricker, A.R., Spiegelhalder, B., and Preussmann, R. (1989). Environmental exposure to preformed nitroso compounds. *Canc Surv* 8(2):251–272.

Tricker, A.R., Ditrich, C., Preussmann, R. (1991). *N*-nitroso compounds in cigarette tobacco and their occurrence in mainstream tobacco smoke. *Carcinogenesis* 12(2): 257–261.

Tricker, A.R., Scherer, G., Conze, C., Adlkofer, F., Pachinger, A., and Klus, H. (1993). Evaluation of 4–(*N*-methylnitrosamino)-4–(3–pyridyl) butyric acid as a potential monitor of endogenous nitrosation of nicotine and metabolites. *Carcinogenesis* 14:1409–1414.

Tricker, A.R. (1997). *N*-Nitroso compounds and man: Sources of exposure, endogenous formation and occurrence in body fluids. *Eur J Canc Prev* 6(3): 226–268.

Trushin, N. and Hecht, S.S. (1999). Stereoselective metabolism of nicotine and tobacco-specific *N*-nitrosamines to 4–hydroxy-4–(3–pyridyl) butanoic acid in rats. *Chem Res Toxicol* 12:164–171.

Tso, T.C. (1990). *Production, Physiology, and Biochemistry of Tobacco Plant.* Institute of International Development & Education in Agricultural and Life Sciences (IDEALS, Inc.), Beltsville, MD.

U.S. Surgeon General (1989). Reducing the health consequences of smoking: 25 years of progress. A report of the Surgeon General. U.S. Department of Health and Human Services, Office on Smoking and Health, Rockville, Maryland.

Wiernik, A., Christakopoulos, A., Johansson, L., and Wahlberg, I. (1995). Effect of air-curing on the chemical composition of tobacco. *Rec Adv Tob Sci* 21:39–80.

Witschi, H., Espiritu, I., Maronpot, R.R., Pinkerton, K.E., and Jones, A.D. (1997). The carcinogenic potential of the gas phase of environmental tobacco smoke. *Carcinogenesis* 18

(11):2035–2042.

Witschi, H., Espiritu, I., Yu, M., and Willits, N.H. (1998). The effects of phenethyl isothiocyanate, N-acetylcysteine, and green tea on tobacco smoke-induced lung tumors in strain A/J mice. *Carcinogenesis* 19:1789–1794.

Witschi, H., Espiritu, I., and Uyeminami, D. (1999). Chemoprevention of tobacco smoke-induced lung tumors in A/J strain mice with dietary myo-inositol and dexamethasone. *Carcinogenesis* 20:1375–1378.

Yahagi, T., Nagao, M., Seino, Y., Matsushima, T., Sugimura, T., and Okada, M. (1977). Mutagenicities of *N*-nitrosamines on Salmonella. *Mut Res* 48:121–130.

Journal of Exposure Analysis and Environmental Epidemiology (1999) 9, 622–635

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The effect of cigar smoking on indoor levels of carbon monoxide and particles

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To provide new information on environmental tobacco smoke (ETS) levels from cigars, we conducted three types of experiments: (1) Measurements of carbon monoxide (CO) during 15 controlled experiments in an office where several cigar brands were machine-smoked; (2) Measurements of CO or respirable suspended particles (RSP) and particle-bound polycyclic aromatic hydrocarbons (PAH) in a residence where two cigars were smoked by a person; and (3) Measurements of CO during two studies at cigar social events (where there were up to 18 cigars being smoked at a time) in which an investigator wore a concealed personal exposure monitor. Average concentrations of CO at the cigar social events were comparable to, or larger than, those observed on a freeway during rush hour traffic. A mass balance model that has been used successfully to predict ETS from cigarettes is used in this paper to obtain CO, RSP, and PAH emission factors (emission rate [mg/min], total mass emitted [mg], and emissions per mass smoked [mg/g]). The calculated emission factors show that the cigar can be a stronger source of CO than the cigarette. In contrast, the cigar may have fewer emissions of RSP and PAH per gram of consumed tobacco than the cigarette, but its size and longer smoking time results in greater total RSP and PAH emissions than for a single cigarette.

Keywords: *carbon monoxide, cigar emissions, cigars, environmental tobacco smoke, human exposure, indoor air quality, polycyclic aromatic hydrocarbons, respirable suspended particles.*

Introduction

In recent years, there has been much concern about exposure to environmental tobacco smoke (ETS), and the U.S. Environmental Protection Agency (USEPA) has classified ETS as a human carcinogen. Indoor cigarette smoking has been the widely accepted source of ETS exposure, and the cigar has received relatively little attention. Consequently, there are difficulties in estimating the health risks of second-hand cigar smoke due to a shortage of air quality and exposure data. Our purpose in the current research is to expand the database of indoor air quality levels from cigars for places where ordinary cigar smokers or aficionados congregate.

In this paper, we characterize the effect of the cigar on indoor air quality by measuring carbon monoxide (CO) concentrations in a variety of indoor settings and using the mass balance model to determine cigar emission factors for CO, respirable suspended particles (RSP), and particle-bound polycyclic aromatic hydrocarbons (PAH). We present results from 16 cigar studies where CO was measured in an office and a residence, and two cigar studies where CO or RSP and PAH were measured in a residence. CO levels measured at two cigar social events (a "cigar smoker" and a "cigar banquet") are also presented in this paper.

1. Abbreviations: USEPA, United States Environmental Protection Agency; ETS, environmental tobacco smoke; RSP, respirable suspended particles; CO, carbon monoxide; ppm, parts per million; PAH, particle-bound polycyclic aromatic hydrocarbons; ϕ, effective air exchange rate ($=\phi_V+\phi_D$) [1/T]; ϕ_V=ventilatory air exchange rate [1/T]; ϕ_D=particle deposition rate [1/T]; τ, residence time (the time required for $z(t)$ to reach 1/e times the original level) [T]; ach, air changes per hour [1/T]; acm, air changes per minute [1/T]; mg, milligrams (10^{-3} g); μm, micrometers (10^{-6} m) [L]; μg, micrograms (10^{-6} g); g, grams [M]; m, meters [L]; l, liters [L^3]; s, seconds [T]; min, minutes [T]; h, hours [T]; $PM_{3.5}$, particulate matter with a diameter of 3.5 μm or less; $PM_{2.5}$, particulate matter with a diameter of 2.5 μm or less; T, duration or averaging time of a given exposure or experimental study [T]; $z(t)$, room concentration at time t [M/L^3]; $n(t)$, number of active sources at time t; v, room volume [L^3]; $\bar{z}$, time-averaged average pollutant concentration [M/L^3]; $g(t)$, total emission rate at time t [M/T]; $\bar{g}$, time-averaged emission rate [M/T]; $\bar{g}_c$, average emission rate per source [M/T]; $\bar{n}$, time-averaged number of sources; T_c, duration of cigar smoking [T]; q, total emissions [M]; q_m, emissions per unit mass smoked [M/M]; g_{eq}, equilibrium emission rate [M/T]; z_{eq}, equilibrium pollutant concentration [M/L^3]; t_{eq}, time required to reach equilibrium [T]; [T^n]=time units; [M^n]=mass units; [L^n]=length units; n=unit dimension (1, 2, or 3).

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Received 23 April 1998; accepted 12 April 1999.

Methods

Instrumentation

As part of our experiments in an office, a residence, and at two cigar social events, we measured instantaneous CO concentrations using a Langan L15 CO Personal Exposure Measurer attached to a Langan DataBear™ digital data logger (Langan Products, San Francisco, CA), which stored concentrations in intervals as short as 10 s. The monitor is equipped with a chemical filter and has been evaluated in previous research (Langan, 1992; Ott et al., 1995).

For the residential study, 2-min averages of RSP concentrations were measured with a TSI Model 8510 piezobalance with a 3.5-μm size-selective inlet (Thermo-Sciences, Inc., TSI, St. Paul, MN). The features and performance characteristics of this instrument are described in the literature (Daley and Lundren, 1975; Sem and Tsurubayashi, 1975; Sem et al., 1977).

Particle-bound ($PM_{2.5}$) PAH were measured in the residence using the EcoChem Model 1002i PAH monitor (West Hills, CA). This monitor is a new instrument designed to measure total PAH on a real-time basis with extremely rapid time response (Burtscher and Schmidt-Ott, 1984; Neissner and Walendzik, 1989; McDow et al., 1990; Chuang and Ramamurthi, 1992; Steiner et al., 1992; Wilson et al., 1994; Agnesod et al., 1996; Ramamurthi and Chuang, 1997a,b). It measures larger molecular weight PAH (above four rings) based on the photoelectric ionization of PAH absorbed on the surface of particles using a mercury vapor lamp with 222 nm ultraviolet wavelength. Neissner and Walendzik (1989) have used the method as a fast-responding and sensitive detection system for cigarette smoke analysis. Wilson et al. (1994) used the EcoChem Model 1002i to measure particle-bound PAH from tobacco smoke (cigarettes). They report that the monitor responds only to fine particles and not to gas-phase PAH, and they obtained reasonably good agreement with chemically based methods when a conversion factor of 1000 ng m^{-3} pA^{-1} was used. Ott et al. (1994) report that PAH monitor readings measured with this same model were highly correlated with RSP concentrations for several types of cigarettes. Buckley and Ott (1996) also used a conversion factor of 1000 ng/m^3=1 pA for PAH concentrations in traffic. In the current study, we report PAH data using a conversion factor of 1000 ng/m^3=1 pA, as suggested by Chuang (1998).

Determining Decay Rates

The present study and previous studies in a tavern (Ott et al., 1996), a residential bedroom (Ott et al., 1995), and two public smoking lounges (Klepeis et al., 1996) have used the cigar as a convenient source of tracer pollutants such as CO and RSP. With appropriate monitoring devices, the overall decay rate ϕ, the ventilatory air exchange rate ϕ_V, and the particle deposition rate ϕ_D can be determined for

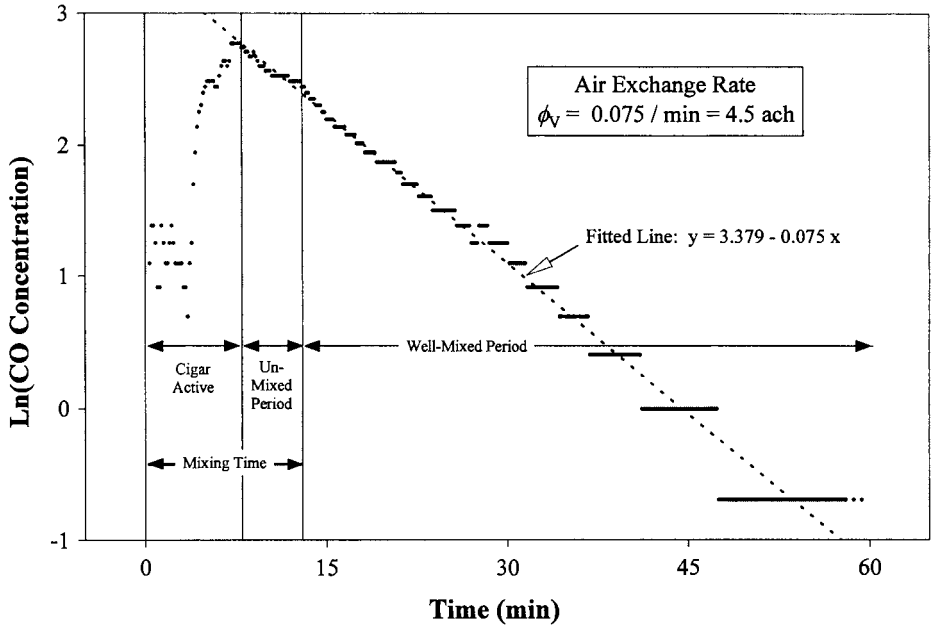

Figure 1. Experimental results from a cigar experiment in an office (experiment O-2) showing how the pollutant decay rate ϕ is determined (in this case, for carbon monoxide). Notice that the unmixed time period (estimated at 5 min) is under 10% of the source-off time period, suggesting that the room can be considered to be well-mixed.

Table 1. Summary of CO emission factors[a] from 15 machine-smoked cigar experiments in a 49.6 m^3 office.

ID	Experiment description[b]	Study duration, T [min]	Cigar duration, T_c [min]	Average CO concentration, $\bar{z}$ [ppm]	Peak CO concentration[c], $z(T_c)$ [ppm]	Average CO emission rate[d], $\bar{g}_c$ [mg/min]	CO emissions per cigar[e], q	Mass smoked[f], m [g]	CO emissions per mass smoked[g], q_m [mg/g]
O-1	1 Sante Fe Fairmont cigar (4/5/96); var ϕ_V	424 (7.1 h)	18	3.6 (4.1 mg/m^3)	14	–	–	6.2	–
O-2	Sante Fe Fairmont cigar (4/6/96); ϕ_V=4.5 ach; see Figures 1 and 2	157 (2.6 h)	7.8	1.7 (2.0 mg/m^3)	16	140	1.1 g	6	190
O-3	1 Sante Fe Fairmont cigar (4/7/96); ϕ_V=0.12 ach	1284 (21 h)	24	7.5 (8.6 mg/m^3)	19	46	1.1 g	6.1	180
O-4	1 Sante Fe Fairmont cigar (4/8/96); ϕ_V=2.1 ach	339 (5.7 h)	20	1.8 (2.1 mg/m^3)	15	61	1.2 g	6	200
O-5	1 Imported Ashton cigar (4/9/96); ϕ_V=1.8 ach	275 (4.6 h)	28	2.5 (2.9 mg/m^3)	15	42	1.2 g	14.3	82
O-6	1 Swisher Sweets cigar (4/22/96); var ϕ_V	167 (2.8 h)	20	4.4 (5.0 mg/m^3)	16	–	–	5.3	–
O-7	1 Swisher Sweets cigar (4/23/96); ϕ_V=0.96 ach	450 (7.5 h)	42	2.4 (2.8 mg/m^3)	9.5	23	980 mg	5.6	180
O-8	1 Dutch Masters El Presidente cigar (4/24/96); ϕ_V=1.9 ach	220 (3.7 h)	15	5.7 (6.5 mg/m^3)	18	–	–	6.4	–
O-9	1 Dutch Masters El Presidente cigar (4/25/96); ϕ_V=2.4 ach	240 (4.0 h)	54	2.1 (2.4 mg/m^3)	7.5	–	–	6.8	–
O-10	1 Dutch Masters El Presidente cigar (4/26/96); ϕ_V=0.06 ach	1440 (24 h)	9	9.2 (11 mg/m^3)	18	84	750 mg	6.6	114
O-11	1 AyC Grenadiers cigar (4/27/96); ϕ_V=0.12 ach	1539 (25.65 h)	10	4.9 (5.6 mg/m^3)	13	86	860 mg	4.9	180
O-12	1 AyC Grenadiers cigar (4/28/96); ϕ_V=3.0 ach	130 (2.2 h)	17	1.7 (2.0 mg/m^3)	7.0	37	630 mg	5.0	130
O-13	1 AyC Grenadiers cigar (4/29/96); ϕ_V=4.5 ach	195 (3.25 h)	12	0.94 (1.1 mg/m^3)	8.0	65	780 mg	4.9	160
O-14	1 AyC cigar (4/29/96); var ϕ_V	87 (1.5 h)	17	0.89 (1.0 mg/m^3)	4.5	–	–	5.1	–
O-15	1 AyC cigar (4/30/96); ϕ_V=0.72 ach	630 (10.5 h)	11	1.3 (1.5 mg/m^3)	8.0	–	–	5.3	–

[a]Calculations of emission factors are based on a single-compartment mass balance model, which assumes uniform mixing.

[b]Experiment descriptions include the type of cigar or cigarette source, the location where smoking took place, the room volume, and the air exchange rate and/or effective air exchange rate, which includes all removal mechanisms (both are in units of ach).

[c]Peak concentrations are the measured concentrations occurring just after the cigar was extinguished (where the theoretical maximum is supposed to occur for the given emission rate, volume, and air exchange rate); however, it is possible that higher concentrations occurred while the cigar was being smoked because of non-uniform mixing.

[d]Average emission rate is the average emission rate over the time the cigar was on; only one cigar was ever active.

[e]Emissions per cigar is the total mass emitted by the cigar (only one cigar was ever active).

[f]Mass smoked is the measured difference between the mass of the unsmoked cigar or cigarette source and the mass after smoking.

[g]Emissions per mass smoked is emissions per cigar divided by mass smoked. 1 ppm=1.145 mg/m^3 at 25°C and 1 atm. Missing cells indicate that conditions were not suitable for the calculation of emission factors (e.g., varying air exchange rate, non-uniform mixing/transient peaks). Constant backgrounds between 0 and 1 ppm were subtracted from each CO time series prior to making calculations. The time interval between all CO measurements was 10 s. See Figure 2 for the O-2 CO time series.

any room. The decay rate ϕ is equal to ϕ_V when obtained from the decay of CO (no surface sorption) and equal to $\phi_D+\phi_V$ when obtained from the decay of RSP, which adheres to surfaces.

In real situations, the room will have a characteristic mixing time τ_{mix}, which is the time it takes for the pollutant to become well-mixed after the cigar is ignited. The decay rate ϕ (sometimes called the effective air exchange rate) is taken as the absolute value of the slope of a least-squares fitted line through the natural logarithm of the pollutant concentration versus time starting after time $t=\tau_{mix}$ (see Figure 1). The background concentration for the levels in the room from sources besides the cigar must be subtracted from each concentration prior to taking its logarithm.

Office Experiments

We conducted 15 experiments (referenced as O-1 through O-15; see summary in Table 1) using single cigars of different types in a vacant, two-person office (volume of 49.6 m^3 and a floor area of 17.0 m^2). CO concentrations were logged in time intervals of 10 s. Background CO concentrations — the levels present in the absence of any cigars — ranged from 0 to 1 ppm. The former occupant of the office regularly smoked cigars during work hours when the office was occupied. The office had a desk but no other furniture. The ventilatory air exchange rate ϕ_V of the office varied from about 0.1 air changes per hour (ach) to about 4.5 ach; it was highest when both the transom over the door and window were open or only the window was open (0.36–4.5 ach), and it was smallest when both the transom and window were closed (about 0.1 ach). There were no forced-air mechanisms or other ventilating systems opera-ting in the office. However, during some experiments when the window was open, the air exchange rate fluctuated widely due to light winds. The duration T of the 15 experiments in the office (Table 1) ranged from 87 min (1.45 h) to 1444 min (24.1 h); for most experiments, concentrations were measured from the time just before the cigar was lighted ($t=0$) until after the time the concentrations decayed to the background level ($t=T$).

Five different, commercially available brands of cigar were used in the office experiments (Sante Fe Fairmont, Imported Ashton, Swisher Sweets, Dutch Masters El Presidente, and Antonio y Cleopatra (AyC) Grenadiers). See Table 2 for the physical characteristics of each cigar. Each cigar was smoked by a smoking machine for between 7 and 40 min (with a mean of 19 min). The smoking machine operated at the center of the room at approximate breathing height; it consisted of a series of tubes that fit snugly around one end of the cigar and a plastic bellows with a small valve from which the mainstream smoke was emitted into the room as the cigar was smoked. Sidestream smoke coming from the burning end of the cigar was also freely emitted into the room. The cigar was smoked by squeezing the bellows at different intervals, and after the cigar was almost completely smoked, it was extinguished by dousing it in a glass of water.

Residential Experiments

Two cigar experiments and one cigarette experiment (referenced as R-1 through R-3; see summary in Table 3) were conducted on two separate days in the 97 m^3 parlor of a San Francisco residence in which a veteran cigar smoker volunteered to smoke two single cigars (a Santona and a Paul Garmirian) and a second volunteer smoked a Marlboro Regular cigarette. The parlor of the residence

Table 2. Physical characteristics of seven different cigars[a] and Marlboro cigarettes[b].

Cigar brand	Experiment(s)	Length [mm]	Mass[c] [g]	Diameter [mm]
Santona	R-1	150	13.2	17
Paul Garmirian	R-2	175	15.4	19
Sante Fe Fairmont	O-1–O-4	152	8.4	16
AyC Grenadiers	O-11–O-15	166	5.9	13
Todo El Mundo (equivalent for Ashton)[d]	O-5	186	16.7	19
Dutch Masters	O-8–O-10	139	7.6	15
Swisher Sweets	O-6–O-7	143	6.0	13
Marlboro (cigarette)	R-3	78 (with a 19 mm filter)	0.83 (with filter); 0.55 (without filter)	6

[a]Cigar characteristics are based on measurements of one cigar (unsmoked).
[b]The mass of an unsmoked Marlboro cigarette (both with and without the filter) was determined by weighing 10 identical cigarettes together and then dividing the total mass by 10; there remained a 10-mm "butt" of tobacco after the filters were cut off.
[c]Measurement errors are about ±0.1 g for cigar masses and about ±0.01 g for cigarettes masses.
[d]A Todo El Mundo cigar of similar size as the Ashton was used for measurements of physical characteristics.

Table 3. Summary of CO, RSP, and particle-bound PAH emission factors[a] from two cigar experiments and one cigarette experiment in a 97 m^3 parlor of a residence.

ID	Experiment description[b]	Study duration, T [min]	Source duration, T_c [min]	Average concentration, $\bar{z}$	Peak concentration[c], $z(T_c)$	Average emission rate[d], $\bar{g}_c$	Emissions per cigar[e], q	Mass smoked[f], m [g]	Emissions per mass smoked[g], q_m
R-1	1 Santona cigar smoked by a person (3/1/97); ϕ=2.0 ach	300 (5 h)	76 (1.3 h)	0.96 ppm CO (1.1 mg/m^3)	3.0 ppm CO	14 mg CO/min	1.1 g CO	8.8	130 mg CO/g
R-2	1 Paul Garmirian cigar smoked by a person (3/9/97); ϕ_v=0.9 ach; ϕ=1.2 ach for RSP; ϕ=1.5 ach for PAH	285 (4.75 h)	90 (1.5 h)	0.16 mg/m^3 RSP; 0.55 μg/m^3 PAH	0.35 mg/m^3 RSP	0.98 mg RSP/min; 0.0042 mg PAH/min	88 mg RSP; 0.38 mg PAH	10.8	8.2 mg RSP/g; 0.035 mg PAH/g
R-3	1 Marlboro cigarette smoked by a person (3/9/97); ϕ=1.3 ach for RSP; ϕ=2.0 ach for PAH	127 (2.75 h)	9	0.065 mg/m^3 RSP; 0.33 μg/m^3 PAH	0.16 mg/m^3 RSP	1.9 mg RSP/min; 0.015 mg PAH/min	17 mg RSP; 0.14 mg PAH	0.4	43 mg RSP/g; 0.34 mg PAH/g

See footnotes under Table 1. See Figure 3 for the RSP and PAH time series from experiments R-2 and R-3.

contained a sofa, chairs, a piano, carpets, drapes, and a fireplace (no fire was lit). See Table 2 for a description of the cigar and cigarette characteristics.

For the first experiment, CO concentrations were measured in 15-s intervals. RSP (2-min averages every 2–3 min) and PAH concentrations (15-s intervals) were measured for the second and third experiments. The cigar experiments lasted 300 and 285 min, respectively, and the volunteer smoked the cigars for 1.3 and 1.5 h, respectively. For the third experiment, which lasted a total of 127 min, the volunteer smoked the cigarette for 9 min in the same parlor. He began smoking the cigarette after elevated particle concentrations caused by the cigar had fallen approximately to background levels (illustrated in Figure 3). The smoking of the cigarette permitted a comparison of cigar and cigarette RSP and PAH levels under the same conditions.

The air exchange rates for the first two experiments were about ϕ=2.0 and ϕ=0.9 ach, respectively, and the RSP decay rate (i.e., the ventilation air exchange rate plus the particle deposition rate) was 1.2 ach for the second experiment (Paul Garmirian cigar) and 1.3 ach for the third experiment (Marlboro cigarette). The parlor doors and windows were closed during all three experiments.

Measurements at Cigar Social Events

In two field experiments, an investigator wearing a concealed CO personal exposure monitor attended social events that featured cigar smoking. The monitor was carried in the inside pocket of a formal dinner jacket so as to not attract the attention of the other guests. Measurements were logged every minute in the first experiment and every 15 s at the second event. The monitor was carried for several hours while traveling to and from each event, so that in-vehicle and outdoor CO concentrations could be compared with those measured during the events.

The first event, a "Cigar Smoker," was held in an elegant private club in suburban San Francisco. This party included food, wine, music, and an author signing his book on cigars. Four different types of cigars were available at the entrance door. The private club was a large house with two adjoining rooms (a large reception hall with a balcony and a food preparation area) measuring 1560 ft^2 (155 m^2) in area, with a volume of 570 m^3. The event's sponsors opened all the doors and windows completely to allow the maximum flow of outdoor air, and the resulting air exchange rate may have exceeded 10 ach. The investigator secretly wearing the monitor smoked the first cigar only partially and then mingled with the other guests in the room and engaged in conversation.

The second concealed monitoring field study took place at a downtown San Francisco restaurant. This particular "Cigar Banquet" (featuring three Cuban cigars per person) was held regularly about every 3 months. The guests received the first cigar when they entered the door. After gathering around the bar to socialize for about an hour prior to being seated for dinner, they were served a second imported cigar. A third cigar was distributed just before dessert.

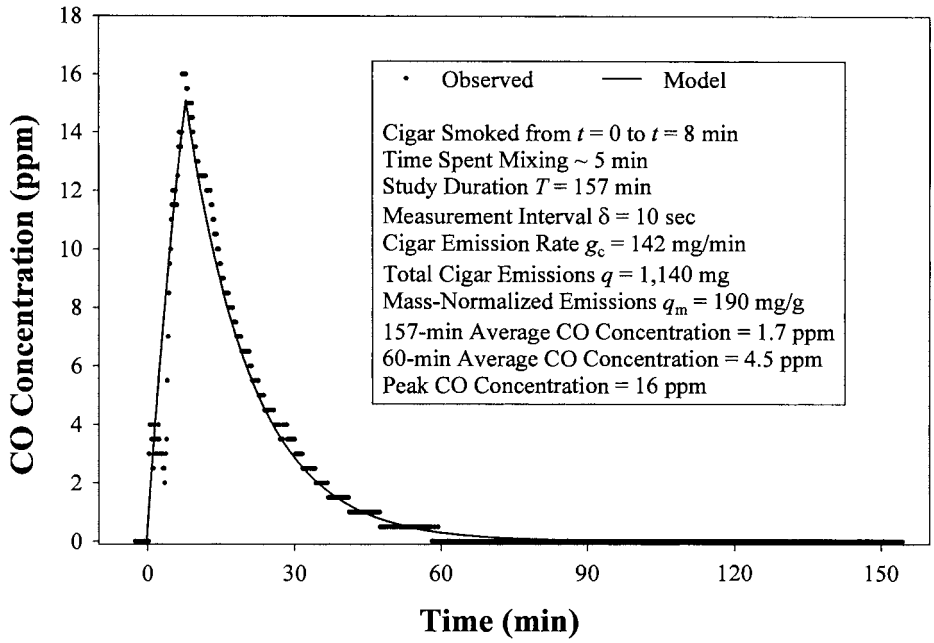

Figure 2. Observed CO concentrations and those predicted by a mass balance model during and after a cigar smoking experiment in a 49.6 m^3 office (experiment O-2). CO emission factors calculated using the model are shown.

Indoor Air Quality Model

Several books discuss the derivation and application of the mass balance equation to predicting indoor air pollutant concentrations (Wadden and Scheff, 1983; Nagda, 1993; Ott, 1995). A variety of scientific papers also discuss its use in determining source emissions and predicting indoor air pollution levels (Alzona et al., 1979; Dockery and Spengler, 1981; Traynor et al., 1982a,b; Repace, 1987). Previous investigators have applied the mass balance equation to predicting ETS from cigarette smoking in indoor settings (Jones and Fagan, 1974; Repace, 1987; Repace and Lowrey, 1980, 1982; Repace et al., 1998; Ott et al., 1992, 1996; Switzer and Ott, 1992; Klepeis et al., 1996), and the results from this modeling usually show good agreement between observed and predicted indoor concentrations due to smoking. A similar mass balance model is used in this paper to calculate emission factors and theoretically predict pollutant concentrations (both time-

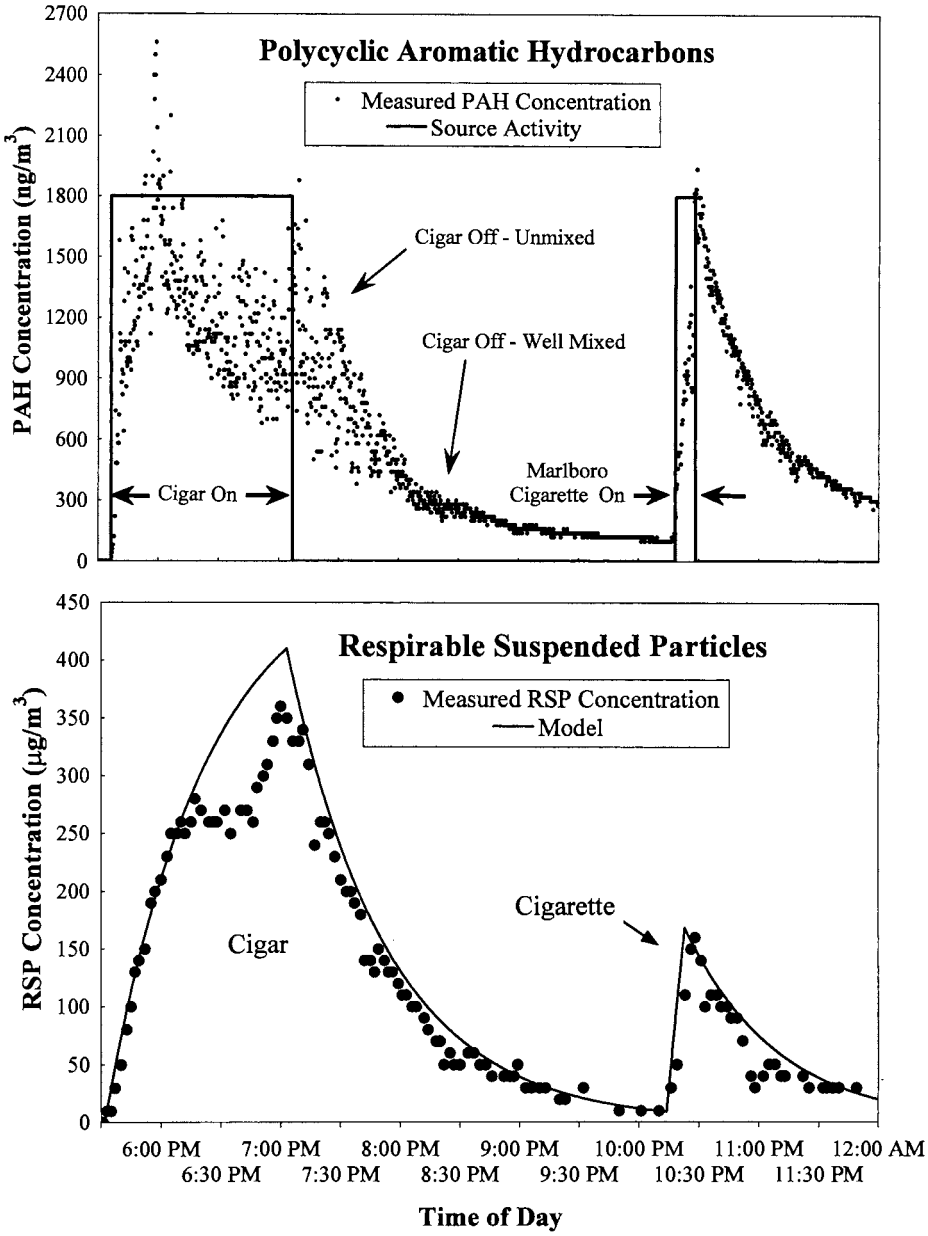

Figure 3. The simultaneous particle-bound PAH and RSP time series measured in the 97 m^3 parlor of a San Francisco residence after a Paul Garmirian cigar and a Marlboro cigarette were smoked one after another by two different persons. The top plot shows the source activity pattern and the bottom plot shows the time series predicted by a model (Experiments R-2 and R-3).

evolving concentrations — i.e., the times series — and time-averaged concentrations).

The model assumes that the air in each location is approximately well-mixed. In a reasonably well-mixed room, the pollutant concentration is uniform across all points in the room at any instant of time. Mage and Ott (1996) propose, based partly on experimental data by Baughman et al. (1994), the classifying of room pollutants as well-mixed if the proportion of time spent in an unmixed state is small in comparison to the overall duration of the study (e.g., the time it takes ETS pollutants to decay to background levels). Several other experimental investigators have demonstrated that reasonably uniform mixing can be achieved for typical exposure time periods under realistic conditions (Drescher et al., 1995; Klepeis et al., 1996; Ott et al., 1996, 1998) such as those under which we performed the experiments described in this paper. For example, Figure 1 shows a visually-estimated unmixed time period of 5 min (for experiment 0–2), which is small compared to the well-mixed time period of over 50 min.

Important emission factors for use in exposure models and in direct comparisons between tobacco and other sources include: (1) the average pollutant emission rate (mass of pollutant emitted per unit time); (2) the total emissions (total mass emitted into the air over some time T); and (3) the mass-normalized emissions (mass of total pollutant emitted per mass of tobacco smoked). Formulae for these three quantities, respectively, are as follows:

$$\overline{g}_{\mathrm{c}} = \overline{z}\phi\nu/\overline{n} \tag{1}$$

$$q = \overline{g}\,T = \overline{g}_{\mathrm{c}}\,\overline{n}\,T = \overline{z}\phi\nu\,T \tag{2}$$

$$q_{\mathrm{m}} = q/m \tag{3}$$

where $\overline{g}_{\mathrm{c}}$ is the average pollutant emission rate [mg/min], $\overline{z}$ is the average pollutant concentration [mg/m^3], ϕ is the effective air exchange rate or decay rate [ach], v is the volume of the room [m^3], $\overline{n}$ is the average number of sources over T [sources], T is the experimental study duration equal to the time period starting when the tobacco source is lit and ending when the pollutant concentration decays to the background level [min], q represents the total emissions from the tobacco source [mg], q_{m} represents the mass-normalized total emissions [mg/g], and m is the total mass of tobacco smoked [mg]. We calculate per-cigar and per-cigarette emission factors in this paper using Equations 1–3. However, we do not calculate emission factors for

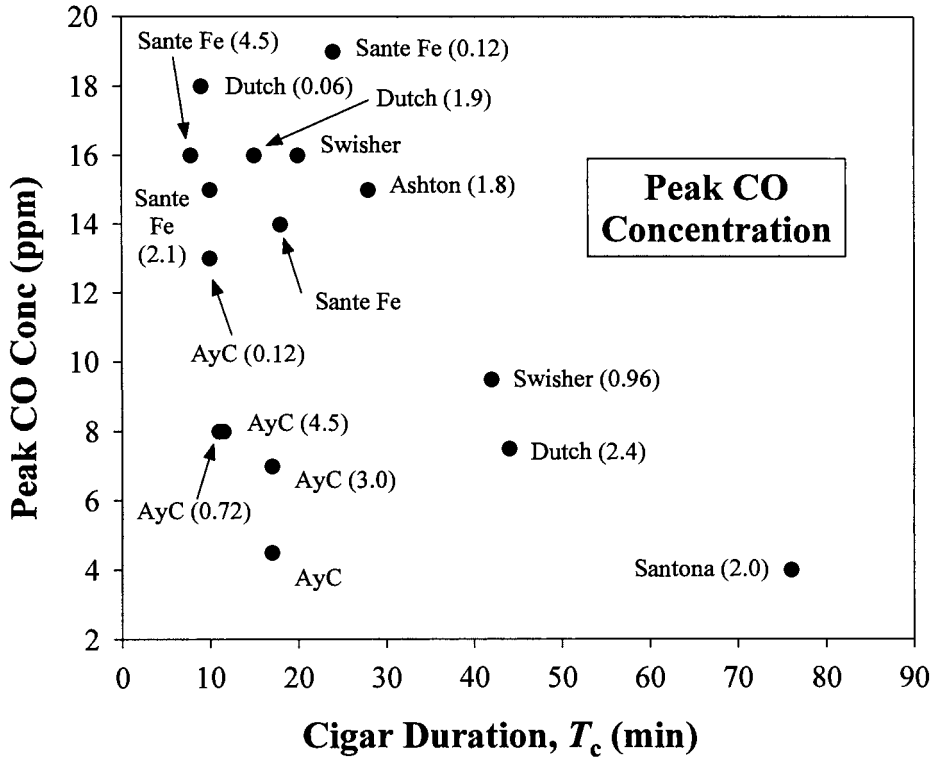

Figure 4. Plot of peak CO concentration versus cigar duration for 16 experiments where CO was measured. Each point is labeled with the cigar brand and the air exchange rate in units of air changes per hour (for experiments where the air exchange rate was determined). Notice that except for the AyC cigars, which have lower total emissions than most of the other cigars, there is a trend towards lower peak CO concentration as the cigar duration increases.

those experiments where conditions were unsuitable, such as when the air exchange rate could not be determined accurately or there appeared to be a large degree of non-uniform mixing.

Figures 2 and 3 (bottom panel) compare the model time series (Ott et al., 1992; Switzer and Ott, 1992; Klepeis et al., 1996) with observed pollutant concentration data for experiments O-2 (CO) and experiments R-2 and R-3 (RSP). Figure 2 also presents CO time series statistics and emission factors determined from the observed CO data. The time-series model requires, as input, the pollutant decay rate ϕ (see above discussion), the room volume v, and the average pollutant emission rate, $\overline{g}_c$, as calculated from the observed pollutant concentration data using Equation 1.

Results and discussion

In the current work, we have not carried out a complete survey of cigar types and smoking situations, but our 16 CO experiments and preliminary experiments with PAH and RSP provide insight into the effect that cigar-smoking can have on indoor air quality. The most useful results are the calculated emission factors (emission rate, total emissions, and emissions normalized by mass smoked), which allow one to gauge the relative strength of different tobacco products and to predict pollutant levels for different smoking scenarios. We also report peak and average concentrations of CO or RSP and PAH in each of the three locations that were studied (office, residence, and social events). Unless stated otherwise, background concentrations were subtracted from all measurements before being used in the calculation of descriptive statistics or emission factors.

Trends in CO Levels

Statistics that are often used in exposure assessment are the average, peak, and steady-state pollutant concentration. A principal difference between cigars and cigarettes is that while a single cigar can be smoked for a very long period (up to an hour or more), cigarettes rarely last more than about 10 min. Different cigar durations T_c affect the peak and average room pollutant concentrations, and it is also possible that smoking the cigar more quickly or more slowly will change its emissions. Two other factors affecting the peak, average, and steady-state concentrations are the physical features of the room (e.g., the volume and the ventilation rate) and the cigar source strength (i.e., the pollutant emission rate).

Since transient peaks sometimes occurred while a cigar was being smoked, we determined the maximum CO concentration at the time when the cigar ended (or just afterwards), which is the time that the theoretical maximum concentration should occur. In Figure 4, the peak CO concentration (3–19 ppm) from the 16 cigar experiments where CO was measured (O-1 through O-15 and R-1) is plotted against the cigar duration (T_c=8–76 min). There is

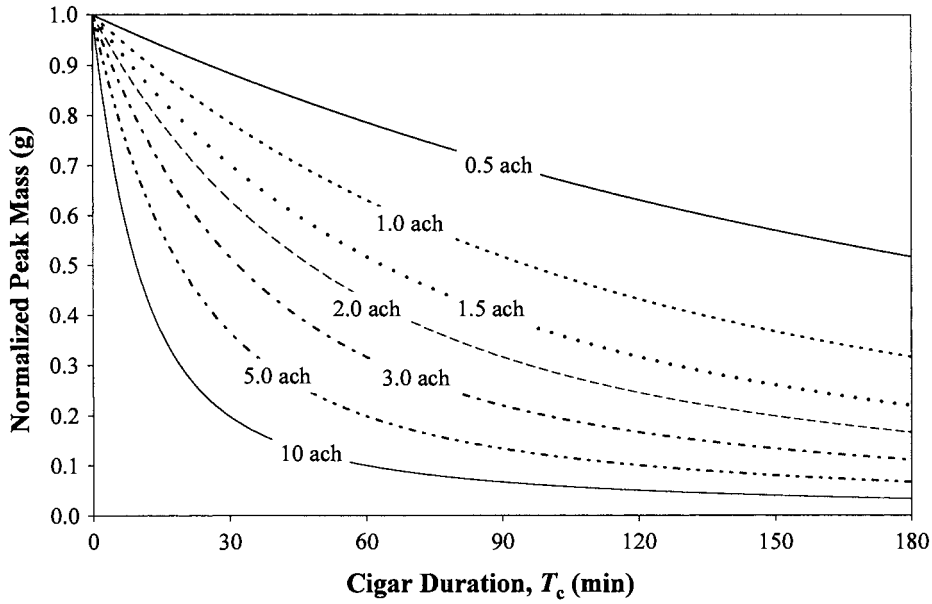

Figure 5. Theoretical curves demonstrating how the pollutant peak airborne mass is affected by the cigar duration T_c and pollutant decay rate ϕ. The total mass emitted by the cigar is held constant and has been normalized to 1 g. Dividing masses by the room volume gives concentrations. The source duration can substantially affect the peak mass, although this effect is non-linear and can sometimes be relatively small for a low decay rate and/or short cigar duration.

a trend toward lower peaks as the cigar duration increases, excluding the AyC brand cigars, which, as we discuss below, have somewhat lower total emissions than most of the other cigars. The low peak for the Santona cigar is probably a result of both the long cigar duration and the larger room volume (97 m^3 for the parlor experiments versus ~50 m^3 for the office experiments). Figure 5 shows, theoretically, how the peak airborne mass changes with both the decay rate (ϕ=0.5–10 ach) and the cigar duration (T_c=1–180 min) when a total of 1 g of pollutant is emitted. The airborne mass, if considered to be distributed in a 1 m^3 room, can be equated to concentration units of grams per cubic meter. For a cigar duration of 30 min, the peak mass ranges from about 90% of the total emitted mass at 0.5 ach to about 20% of the total emitted mass at 10 ach. Obviously, if the mass is emitted nearly instantaneously (e.g., over 1 min or less), then the peak mass will be equal to the total mass emitted.

In contrast with the observed peak concentrations, which ranged from 3 to 19 ppm, the 60-min time-averaged CO concentrations (calculated from when the cigar was first ignited) seem to depend in a more distinct way on the decay rate, but do not depend as strongly on cigar duration. When the air exchange rate is greater than or equal to 2 ach, the observed average CO concentrations are all below 8 ppm (Figure 6; unfilled circles), whereas for air exchanges below 2 ach, all except one of the observed average CO concentrations are above 8 ppm (Figure 6; filled circles). The theoretical pollutant time series in Figure 7, calculated for constant pollutant decay rates ϕ and 1 g of total emissions, shows that the 180-min average airborne pollutant mass does not change much even when the cigar duration is increased from 1 to 120 min. Regardless of the value of T_c, the average airborne mass is around 30% of the total mass emitted at 1 ach and 7% of the total mass emitted at 5 ach.

When a cigar is smoked for an extended period of time, it is possible to reach a characteristic equilibrium concentration for a given emission rate and pollutant decay rate. Unless the cigar is smoked for a very long time, the concentrations may never quite reach this level, but it provides an upper-bound to the maximum pollutant concentration for the given room conditions and pollutant emission rate. The modeled equilibrium concentration equals the equilibrium emission rate g_{eq} divided by the volume and the decay rate: $z_{eq}=g_{eq}/v\phi$. However, the time, t_{eq}, that it takes to reach 99% of the equilibrium concentration depends only on the pollutant decay rate: $t_{eq}=-\ln(0.01)/\phi$. For a pollutant decay rate of 1 ach (fairly typical for a residence), t_{eq}=4.6 h.

Calculated Emission Factors

The cigars that were studied have average CO emission rates $\overline{g}_c$ ranging from 14 to 140 mg/min and total emissions

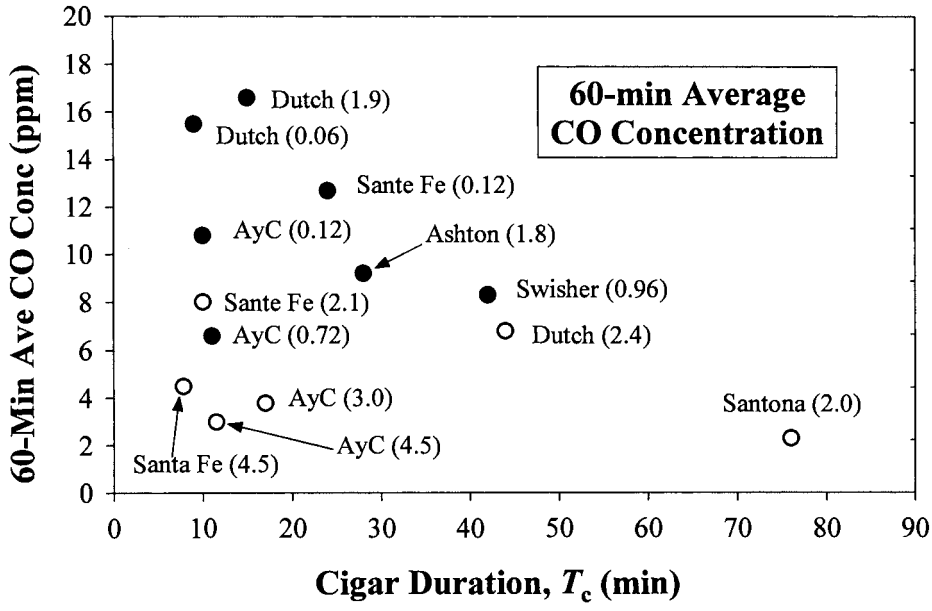

Figure 6. Plot of the 60-min average CO concentration (calculated from the time the cigar begins) versus cigar duration for 13 experiments where CO was measured and the air exchange rate was determined. The unfilled circles designate data from experiments with an air exchange greater than or equal to 2 ach and filled circles designate data for experiments with an air exchange rate less than 2 ach. Notice that all of the average CO concentrations at higher air exchange rates are below about 8 ppm, whereas six of seven of the average CO concentrations at lower air exchanges rate are above 8 ppm. Averages over the entire study duration T (87–1440 min) are given in Tables 1 and 3.

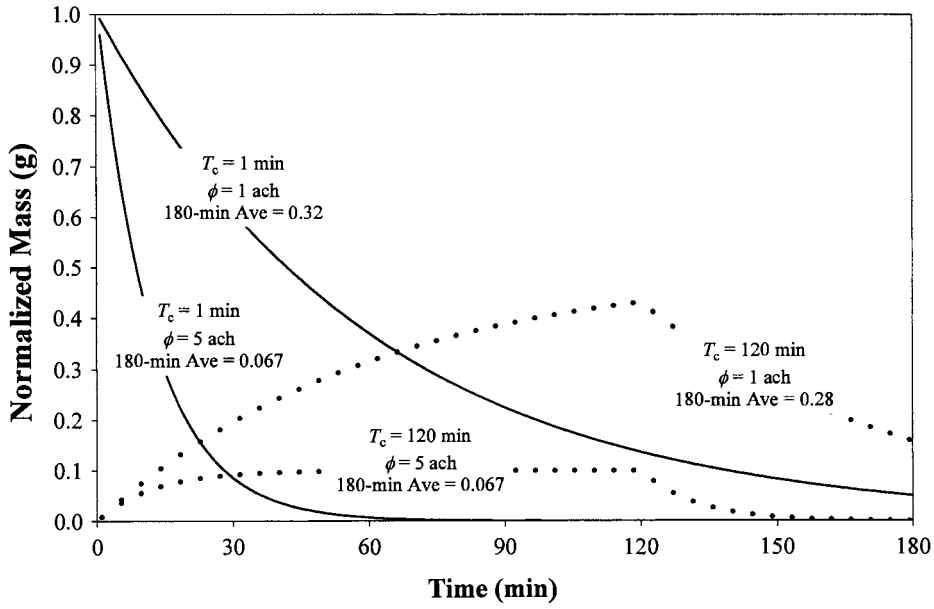

Figure 7. Theoretical curves demonstrating how the average airborne pollutant mass is affected by the cigar duration T_c and pollutant decay rate ϕ. The total mass emitted by the cigar is held constant and has been normalized to 1 g. Dividing masses by the room volume gives concentrations. Notice that for a given time period (T = 180 min) and decay rate (ϕ = 1 or 5 ach), the cigar duration does not greatly affect the average room concentration.

between 630 and 1200 mg/cigar, with the Santona having the lowest emission rate (14 mg/min) and the AyC and Dutch Master cigars having the lowest total emissions (630–860 mg/cigar). See Tables 1 and 3 for summaries of emission factor results. The Sante Fe cigars have the greatest mass-normalized emissions at 180–200 mg/g, and the Ashton (82 mg/g) and the Dutch Masters (114 mg/g) have the smallest. Although the Ashton has one of the lowest emission rates and the smallest mass-normalized emissions, its large (unsmoked) mass (14.3 g) causes its total emissions to be as high as any of the other cigars that were studied (1.2 g).

The Paul Garmirian cigar, which was smoked for 90 min, emitted a total of 88 mg of RSP and 0.38 mg of PAH (Table 3). The emission rates for RSP and PAH were 0.98 and 0.0042 mg/min, respectively, and the RSP and PAH mass-normalized emissions q_m were 8.2 and 0.035 mg/g, respectively. In contrast, the Marlboro cigarette that was smoked under identical conditions had RSP and PAH mass-normalized emissions that were five and ten times higher (43 and 0.34 mg/g), and RSP and PAH emission rates that were two and four times as large as the cigar (1.9 and 0.015 mg/min). However, the total RSP and PAH emissions of the cigar are five and three times higher, respectively, than those of the cigarette (17 and 0.14 mg), which is not that large a difference considering that the mass of cigar tobacco smoked is about twenty-seven times larger than the mass of cigarette tobacco that was smoked.

Comparison to Published Cigarette Emission Factors

As reported in the literature (Rosanne and Owens, 1969; Rickert et al., 1984; Ott et al., 1992), total CO emissions from cigarettes are typically between 40 and 70 mg for sidestream smoke. If the mass of tobacco smoked is about 0.4 g (as it was for one of our experiments), then, neglecting mainstream emissions, the CO emissions per mass smoked would be 100–175 mg/g. If more of the cigarettes were smoked (e.g., 0.55 g; see Table 2), then the CO emissions per mass smoked could be as low as 70–130 mg/g. Our results show that CO emissions per mass smoked for cigars are between about 100 and 200 mg/g (Table 1) and Ott et al. (1992) report an average CO emission rate of 9.4 mg/min per cigarette, which is lower than the 14–140 mg/min average emission rates that we found for cigars. Thus, cigars can be a much stronger source of CO than cigarettes.

For RSP, our results show that mass-normalized emissions can be five times larger for cigarettes than for cigars (40 versus 8 mg/g), and the PAH emissions for a cigarette can be about ten times that of a cigar (0.34 versus 0.035 mg/g). Leaderer and Hammond (1991) report 48 mg/g of RSP emitted from a cigar (a cigarillo according to Leaderer, personal communication) and 23–35 mg/g emitted from 12 different cigarettes — suggesting that some types of cigars may have larger RSP mass-normalized emissions than cigarettes. Daisey et al. (1994) report average RSP ($PM_{2.5}$) emissions of 12.4 mg/g over six

types of machine-smoked cigarettes, a result which is closer to our value for a cigar.

CO Levels at Two Cigar Social Events

At the Cigar Smoker (the first cigar-smoking event that we studied), about 50 persons were present (12 women and 38 men), and indoor CO concentrations ranged between 5 and 11 ppm, giving an indoor average of about 6 ppm (see Figure 8a). The highest CO concentrations occurred on the upstairs balcony of the main hall, suggesting that CO concentrations were higher near the ceiling than at the floor. If we adjust the observed CO concentrations by subtracting the ambient CO levels of 1.5 ppm measured outside the building while walking on sidewalks, then about 18 active smokers in the hall contributed 4.5 ppm, approximately the same level as measured on the freeway while driving to the event. The high air exchange rate caused by the wide-open doors and windows probably reduced the CO concentrations in the building considerably.

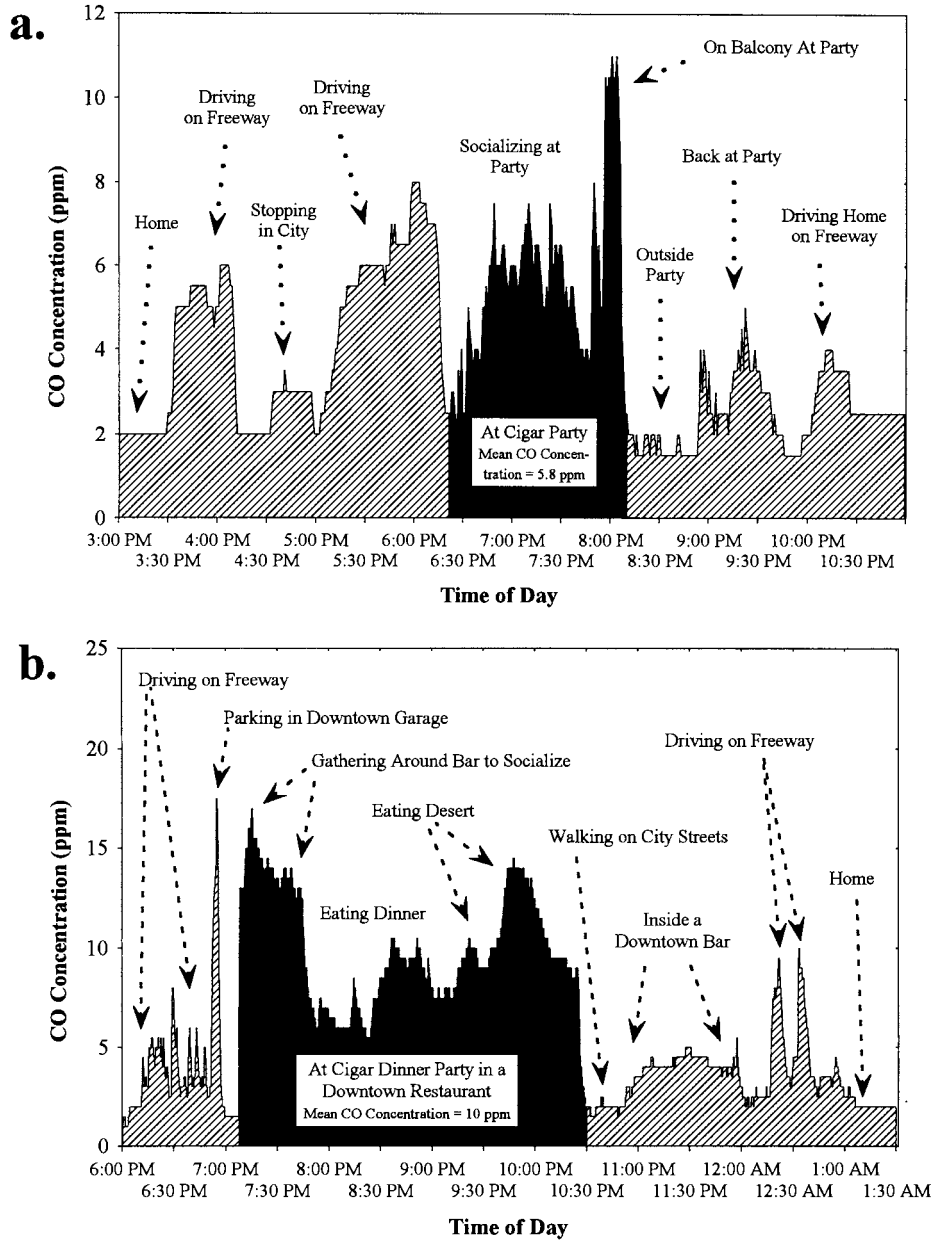

Figure 8. CO concentrations measured using a concealed personal exposure monitor before, during, and after (a) a "cigar smoker" in a San Francisco club and (b) a "cigar banquet" in a San Francisco restaurant. Background levels have not been subtracted from the CO levels shown in the figure.

At the Cigar Banquet (the second event that we studied), the outdoor ambient CO concentration was about 1 ppm, and indoor levels during the first hour when about 24 smokers were at the bar were 13–17 ppm (see Figure 8b). At 7:45 pm h, the patrons were seated for dinner at individual tables of four to six persons each for a three-course dinner, which included cigars. The investigator was seated with five other persons, all of whom smoked cigars during dinner. Overall, more than 100 cigars were smoked during the dinner party. The indoor CO concentration averaged over the 3-h-and-20-min visit was 10 ppm, and about 75% of the 40 persons present was smoking cigars at any instant of time. Based on measurements outdoors on downtown sidewalks before and after the event, ambient CO concentrations were found to be about 1 ppm, so the indoor CO concentration caused by cigar smoking in the restaurant was about 9 ppm. If the cigar dinner had lasted more than 8 h, then indoor CO concentrations might have exceeded the USEPA National Ambient Air Quality Standard (NAAQS) designed to protect public health (9 ppm for 8 h). After leaving the restaurant where the cigar banquet was held, the investigator walked to a San Francisco bar where several cigarettes were being smoked but no cigars were present; indoor CO levels were much lower than at the cigar banquet (Figure 8b).

CO concentrations recorded on the freeway while driving to and from both cigar social events averaged approximately 4.5 ppm. This level is approximately the same as the average in-traffic CO concentration of 4.6 ppm measured on 96 trips during a year-long study of an urban arterial highway in the Bay Area (Ott et al., 1993).

Conclusions

This paper reports cigar emission factors and peak and average concentrations for CO, RSP, and PAH from 17 new cigar experiments. From our results, it appears that for a given mass smoked, cigars can emit larger amounts of CO than cigarettes. This point is illustrated from the CO levels we measured at two cigar social events that are comparable to CO levels on a busy freeway. Cigar smoking can elevate indoor CO concentrations in a restaurant considerably, even when the doors are wide open and the ventilation system is fully operating. Even though cigars appear to emit either less or comparable amounts of RSP and PAH per given mass than cigarettes, cigars are more massive than cigarettes, and they are smoked for much longer periods of time (10 min or less for a cigarette versus as much as an hour or more for cigars). Thus, total emissions from a fully smoked cigar can be two to three times that of a cigarette for PAH, five times that of a cigarette for RSP, and nine to thirty times that of a cigarette for CO (630–1200 mg CO/cigar versus 40–70 mg CO/cigarette). A bystander who is present during the smoking of a cigar receives a much higher exposure to CO, RSP, and PAH than would likely occur for a single cigarette.

Acknowledgments

The authors thank Mr. Lee Langan (for being our "real cigar smoker") and his wife Karine (for allowing an invasion of equipment and investigators into her home). Appreciation is extended to Donald Shopland of the National Cancer Institute. This research was partially funded by the Tobacco Related Disease Research Program of the State of California grant no. 6RT-0118.

References

Agnesod G., De Maria R., Fontana M., and Zublena M. Determination of PAH in airborne particulate: comparison between off-line sampling techniques and an automatic analyzer based on a photoelectric aerosol sampler. *Sci. Total Environ.* 1996: 189/190: 443–449.

Alzona J., Cohen B.L., Rudolph H., Jow N., and Frohliger J.O. Indoor–outdoor relationships for airborne particulate matter of outdoor origin. *Atmos. Environ.* 1979: 15: 55–60.

Baughman A.V., Gadgil A.J., and Nazaroff W.W. Mixing of a point source pollutant by natural convection flow within a room. *Indoor Air* 1994: 4: 114–122.

Buckley T.J., and Ott W.R. Demonstration of real-time measurements of PAH and CO to estimate in-vehicle exposure and identify sources. Proceedings of the International Symposium, Measurement of Toxic and Related Air Pollutants. USEPA and Air and Waste Management Association, Research Triangle Park, NC, 1996.

Burtscher H., and Schmidt-Ott A. Surface enrichment of soot particles in photoelectrically active trace species. *Sci. Total Environ.* 1984: 36: 233–238.

Chuang J.C. Battelle Memorial Institute, personal communication. Annual Meeting of the International Society of Exposure Analysis, Boston, MA, August 18, 1998.

Chuang J.C., and Ramamurthi M. Evaluation of a Gossen, GMBH Model PAS 1000i photoelectric aerosol sensor for real-time PAH monitoring. Final report under contract no. 68-DO-007 from Battelle Memorial Institute, Columbus, OH, to Nancy K. Wilson, Project Officer, USEPA, Atmospheric Research and Exposure Assessment Laboratory, Research Triangle Park, NC, 1992.

Daisey J.M., Mahanama K.R.R., and Hodgson A.T. Toxic volatile organic compounds in environmental tobacco smoke: emission factors for modeling exposures of California populations. Final report, contract no. A133-186 to Lawrence Berkeley Laboratory, Berkeley, CA, California Air Resources Board, Sacramento, CA, 1994.

Daley P.S., and Lundren D.A. The performance of piezoelectric crystal sensors used to determine aerosol mass concentrations. *Am. Ind. Hyg. Assoc. J.* 1975: 26: 518–532.

Dockery D.W., and Spengler J.D. Indoor–outdoor relationships of respirable sulfates and particles. *Atmos. Environ.* 1981: 15: 335–343.

Drescher A.C., Lobascio C., Gadgil A.J., and Nazaroff W.W. Mixing of a point-source indoor pollutant by forced convection. *Indoor Air* 1995: 5: 204–214.

Jones R.M., and Fagan R. Application of mathematical model for the buildup of carbon monoxide from cigarette smoking in rooms and houses. *ASHRAE J.* 1974: 16: 49–53.

Klepeis N.E., Ott W.R., and Switzer P. A multiple-smoker model for predicting air quality in public lounges. *Environ. Sci. Technol.* 1996: 30: 2813–2820.

Langan L. Portability in measuring exposure to carbon monoxide. *J. Expos. Anal. Environ. Epidemiol.* 1992: 1 (suppl): 223–289.

Leaderer B.P., and Hammond S.K. Evaluation of vapor-phase nicotine and respirable particle mass as markers for environmental tobacco smoke. *Environ. Sci. Technol.* 1991: 25: 770–777.

Mage D.T., and Ott W.R. Accounting for nonuniform mixing and human exposure in indoor microenvironments. In: (Tichenor B.A., Ed.), Characterizing Sources of Indoor Air Pollution and Related Sink Effects. West Conshohocken, PA, ASTM STP 1287, PCN 04-12870-17, 1996, pp. 263–269.

McDow S.R., Giger W., Burtscher H., Schmitt-Ott A., and Siegman H.C. Polycyclic aromatic hydrocarbons and combustion aerosol photoemission. *Atmos. Environ.* 1990: 24A: 2911–2916.

Nagda N.L. (Ed.). Modeling of Indoor Air Quality and Exposure, ASTM STP 1205. American Society for Testing and Materials, West Conshohocken, PA, 1993.

Neissner R., and Walendzik G. The photoelectric aerosol sensor as a fast-responding and sensitive PAH detection system for cigarette smoke analysis. *Fresenius Z. Anal. Chem.* 1989: 333: 129–133.

Ott W.R. Environmental Statistics and Data Analysis. Lewis Publishers, CRC Press, Boca Raton, FL, 1995.

Ott W.R., Langan L., and Switzer P. A time series model for cigarette smoking activity patterns: model validation for carbon monoxide and respirable particles in a chamber and an automobile. *J. Expos. Anal. Environ. Epidemiol.* 1992: 2: 175–200.

Ott W.R., Switzer P., and Willits N. Trends of in-vehicle CO exposures on a California arterial highway over one decade. Paper No. 93-RP-116B.04 presented at the 86th Meeting of the Air and Waste Management Association, Denver, CO, 1993.

Ott W.R., Wilson N.K., Klepeis N.E., and Switzer P. Real-time monitoring of polycyclic aromatic hydrocarbons and respirable suspended particles from environmental tobacco smoke in a home. Proceedings of the International Symposium, Measurement of Toxic and Related Air Pollutants, Durham, NC, Report No. VIP-39, Air and Waste Management Association, Pittsburgh, PA, NTIS PB-94-20956, 1994.

Ott W.R., Vreman H.J., Switzer P., and Stevenson D.K. Evaluation of electrochemical monitors for measuring carbon monoxide concentrations in indoor, in-transit, and outdoor microenvironments. Presented at the International Symposium on the Measurement of Toxic and Related Air Pollutants of the Air and Waste Management Association, Research Triangle Park, NC, 1995.

Ott W.R., Switzer P., and Robinson J. Particle concentrations inside a tavern before and after prohibition of smoking: evaluating the performance of an indoor air quality model. *J. Air Waste Manage. Assoc.* 1996: 46: 1120–1134.

Ott W.R., Klepeis N.E., and Switzer P. Modeling Environment Tobacco Smoke in the Home using Transfer Functions. Paper Number 95-WP84B.03 Presented at the 88th Annual Meeting of the Air and Waste Management Association. San Antonio, TX, June 18–23, 1995.

Ramamurthi M., and Chuang J.C. Field and laboratory evaluation of a real-time PAH analyzer. EPA Report no. EPA/600/R-97/034, prepared by Battelle Memorial Institute, Columbus, OH, for Nancy K. Wilson, Project Officer, USEPA, National Exposure Research Laboratory, Research Triangle Park, NC, NTIS Report No. PB97-176838, 1997a.

Ramamurthi M., and Chuang J.C. Evaluation of a photoelectric aerosol sensor for real-time PAH monitoring. EPA Report No. EPA/600/R-97/035, prepared by Battelle Memorial Institute, Columbus, OH, for Nancy K. Wilson, Project Officer, USEPA, National Exposure Research Laboratory, Research Triangle Park, NC, NTIS Report No. PB97-147938, 1997b.

Repace J.L. Indoor concentrations of environmental tobacco smoke: models dealing with effects of ventilation and room size. In: (O'Neill I., Brunemann K., Dodet B., and Hoffman D., Eds.), Environmental Carcinogens — Selected Methods of Analysis — Passive Smoking, Vol. 9, Chap. 3. IARC Scientific Publications No. 81, International Agency for Research on Cancer, World Health Organization, United Nations Environment Program, Lyon, France, 1987.

Repace J.L., and Lowrey A.H. Indoor air pollution, tobacco smoke, and public health. *Science* 1980: 208: 464–474.

Repace J.L., and Lowrey A.H. Tobacco smoke, ventilation, and indoor air quality. *ASHRAE Trans.* 1982: 88: 894–914.

Repace J.L., Jinot J., Baynard S., Emmons K., and Hammond S.K. Air nicotine and saliva cotinine as indicators of passive smoking exposure and risk. *Risk Anal.* 1998: 18: 71–83.

Rickert W.S., Robinson J.C., and Collishaw N. Yields of tar, nicotine, and carbon monoxide in the sidestream smoke from 15 brands of canadian cigarettes. *Am. J. Public Health* 1984: 74: 228–231.

Rosanno A.J., and Owens D.F. Design procedures to control cigarette smoke and other air pollutants. *ASHRAE Trans.* 1969: 75: 93–102.

Sem G.J., and Tsurubayashi K. A new mass sensor for respirable dust measurement. *Am. Ind. Hyg. Assoc. J.* 1975: 36: 791–800.

Sem G.J., Tsurubayashi K., and Homma G.J. Performance of the piezoelectric microbalance respirable aerosol sensor. *Am. Ind. Hyg. Assoc. J.* 1977: 38: 580–588.

Steiner D., Burtscher H., and Gross H. Structure and disposition of particles from a spark-ignition engine. *Atmos. Environ.* 1992: 26A: 997.

Switzer P., and Ott W.R. Derivation of an indoor air averaging time model from the mass balance equation for the case of independent source inputs and fixed air exchange rates. *J. Expos. Anal. Environ. Epidemiol.* 1992: 2: 113–135.

Traynor G.W., Anthon D.W., and Hollowell C.D. Technique for determining pollutant emissions from a gas-fired range. *Atmos. Environ.* 1982: 16: 2979–2988.

Traynor G.W., Apte M.G., Dillworth J.F., Hollowell C.D., and Sterling E.M. The effects of ventilation on residential air pollution due to emissions from a gas-fired range. *Environ. Int.* 1982: 8: 447–452.

Wadden R.A., and Scheff P.A. Indoor Air Pollution: Characterization, Prediction, and Control. John Wiley and Sons, New York, NY, 1983.

Wilson N.K., Barbour R.K., Chuang J.C., and Ramamurthi M. Evaluation of a real-time monitor for fine particle-bound PAH in air. *Polycyclic Aromatic Compounds* 1994: 5: 167–174.

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ScienceDirect
Regulatory Toxicology and Pharmacology 48 (2007) 320–330
Regulatory Toxicology and Pharmacology
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A comparative study of the mutagenicity of various types of tobacco products ☆

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Received 21 February 2007
Available online 21 May 2007

Abstract

Toxicological data are an important aspect of tobacco product characterization. In this study, TPM (Total Particulate Matter) (three replicates) was collected from cigarettes [five brands, ISO conditions: puff volume, 35 mL; duration, 2 s; interval, 60 s (35/2/60)], cigars (two brands, 45/2/30), cigarillos (two brands, 35/2/60), bidis (two brands, 45/2/30), and pipe tobacco (two brands, 50/2/12). TPM was extracted from the Cambridge filter pad using dimethyl sulfoxide (DMSO). Smokeless tobacco (ST) (six brands) was extracted with DMSO using an ultrasonic homogenizer. Both types of extracts were filtered and stored at −80 °C. All extracts were analyzed for humectants, water and nicotine. Mutagenic activity was assessed per OECD guideline 471 using *Salmonella typhimurium* TA98+S9 and TA100+S9. TA98+S9 response (specific activity expressed as revertants/mg nicotine) was greatest for the cigarette fabricated with dark, air-cured tobaccos. Average product responses with TA98+S9 based on nicotine and relative to cigarettes (excluding dark tobacco) were cigars, 242%; cigarillos, 238%; bidis, 91%; and pipe tobacco, 44%. ST response was not significant for TA98+S9. Corresponding values for TA100+S9 were cigars, 189%; cigarillos, 155%; pipe tobacco, 130%; bidis, 114% and ST, 34%. ST TA100+S9 response ranged from a low of 501 to a high of 8547 revertants/mg nicotine, depending on ST composition.

Keywords: Ames; *Salmonella typhimurium*; Bidis; Chemistry; Chewing; Cigarettes; Cigars; Mutagenicity; Smoke; Snuff; Tobacco

1. Introduction

In the latter decades of the twentieth century, US cigarette consumption began to decline on account of increased health concerns and taxes. Conversely, sales of other smoking products such as cigars increased after declining for many years (Wehlburg, 1999). Sales of smokeless tobacco products, which had also declined, increased, particularly sales of wet snuff (Surgeon General, 1986). Part of this increase in the sales of noncigarette tobacco products was believed due to consumer perceptions that smokeless tobaccos and cigars were safer than cigarettes. The US Congress mandated warning labels on smokeless tobacco products in 1986. One of those warnings was, "This product is not a safe alternative to smoking" (CDC, 2000). The basis for such warnings was evidence that use of smokeless tobacco products had been associated with oral cancer and other diseases and such products were addictive (Surgeon General, 1986). Warnings were put on cigar products in 2000 (CDC, 2000).

While most experts see little difference in the health risks associated with smoking noncigarette tobacco products versus smoking cigarettes, there has been increased debate about the health risks associated with smokeless tobacco products over the last decade as exemplified by Nilsson's risk assessment on snuff dipping (Nilsson, 1998). One of the reasons for debate has been that there are many different kinds of smokeless tobacco products used worldwide. Traditionally, three types of smokeless tobacco products

☆ Presented, in part, at the 58th Tobacco Science Research Conference, 19–22 September 2004, Winston-Salem, North Carolina, USA.

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doi:10.1016/j.yrtph.2007.05.003

have been sold in North America: (1) chewing tobacco (looseleaf, plug, twist); (2) dry snuff; and (3) wet snuff (Surgeon General, 1986; Wahlberg and Ringberger, 1999). Each of these three types of products is different, chemically and physically, from each other and each of them differs in many respects from smokeless tobacco products made outside North America (Nilsson, 2006, 1998; Rodu and Jansson, 2004). Some have advocated use of smokeless tobacco products as a substitute for cigarettes for those who cannot or will not stop smoking (Levy et al., 2004). However, others feel that the health risks of such a proposition are too high and that only medicinal nicotine should be used (Hatsukami et al., 2004). While there is considerable epidemiological evidence supporting the use of certain smokeless tobacco products as has been pointed out by experts in the field (Rodu and Godshall, 2006; Nilsson, 2006; Rodu and Jansson, 2004; Bates et al., 2003), there is a lack of bioassays to distinguish smokeless tobacco products deemed less hazardous from those that could be more hazardous. In addition, it would also be desirable to have bioassays to compare the hazards of smokeless tobacco products with smoking products to assist public health officials in making policies on use of smokeless tobacco products. Furthermore, it would be desirable to be able to put the results of such bioassays on a common metric to compare all tobacco products whether smoking or not.

There are no indications that use of smokeless tobacco products has been associated with smoking-related nonneoplastic lung disease such as chronic obstructive pulmonary disease (Anczak and Nogler, 2003). Furthermore, there is no environmental tobacco smoke (ETS) generated by use of smokeless tobacco. The debate has been focused on the relationships between smokeless tobacco use and various cancers. At least one expert has related all associations between smokeless tobacco and cancer to the tobacco specific nitrosamines (TSNAs) in the products (Nilsson, 2006). However, the TSNA levels in contemporary, commercial smokeless tobacco products as well as mainstream cigarette smoke are thought to be too low to contribute to the mutagenicity as measured by the Ames assay especially in the presence of nicotine (Brown et al., 2001; Grasso et al., 1996; Deaton, 1987). On the other hand, extracts of smokeless tobacco products have been found to be mutagenic (Nair et al., 2004; Niphadkar et al., 1996; Stamm et al., 1994; Jansson et al., 1991; Guttenplan, 1987). Some authors have reported correlations between results of various *in vitro* assays for mutagenicity such as the Ames *Salmonella*/microsome mutagenicity assay with the results on rodent carcinogenicity studies (Mortelmans and Zeiger, 2000; Kim and Margolin, 1999a). Thus, there have been numerous reports over the years of the Ames assay being used to determine and compare the mutagenicity of tobacco smoke condensates as noted in the reviews by DeMarini (2004), Massey (2002). In addition, and as noted above, several researchers had determined the Ames activity of extracts of smokeless tobacco products. However, none of these studies had been designed to compare conventional cigarette products, with other contemporary smoking products as well as contemporary smokeless tobacco products available in the North American market. In the study reported herein, a number of smoking and smokeless tobacco products were obtained and characterized both chemically and with the Ames assay. Furthermore, the results from the Ames assay have been put on a per-unit nicotine basis to permit comparison of smoking and smokeless products on a per unit nicotine basis.

2. Experimental

Smoking products evaluated included several types of filtered cigarettes (dark air-cured, blended, flue-cured), cigarillos, cigars, bidis (made in India), and pipe tobacco. Three types of smokeless tobacco products were evaluated: (1) pouched and loose wet snuff typical of products sold in US and Canada; (2) tableted and loose dry snuff reportedly made with specially cured, low-nitrosamine tobaccos; and (3) two types of smokeless tobacco products from India. The first of these was a gutkha, which is a sweetened mixture of tobacco, betel nut, lime, catechu and other ingredients popular in India. The second product is a chewing tobacco known as zarda. Zarda is a mixture of tobacco flakes, silver leaves, aromatic spices, and synthetic flavors. Zarda is generally chewed in various combinations with other materials such as betel quid or a mixture of areca nut and lime. Tobacco products were obtained at retail from domestic and international markets except for the CIM-7 (Canadian Industry Monitor, 100% flue-cured tobacco by Canadian Tobacco Manufacturers Council, Ottawa, Canada and provided free of charge) and the Kentucky KR2R4F reference cigarettes [Kentucky Tobacco Research and Development Center (formerly known as the Kentucky Tobacco and Health Institute), Lexington, KY 40546–0236, USA]. On receipt, they were stored at 4 °C until laboratory use. Smoking products were conditioned according to ISO 3402 prior to analyses (ISO, 1999). Cigarettes and cigarillos were smoked according to ISO 4387 (35 mL puff, 2-s puff duration, 60-s puff interval) using a Borgwaldt RM-20/CS smoking machine (ISO, 2000). More intensive smoking conditions were not used as they tend to give reduced mutagenicity values when values are expressed as revertants per unit weight of TPM (Roemer et al., 2004; Rickert et al., 2002).

Cigars (using Cerulean SM-400) and bidis (using Borgwaldt RM-20/CS) were smoked under similar conditions except that the puff volume was 45 mL and the puff interval was 30 s (Field et al., 2001). Pipe tobaccos were smoked according to the procedure described by Joza and co-workers with a 50 mL puff of 2-s duration taken every 12 s (Joza et al., 2001). Glycerin and propylene glycol contents of the TPM were measured in addition to water and nicotine. For the mutagenicity studies, TPM was dispersed in DMSO at a concentration of 10 mg TPM/mL, the mixture filtered

through sterile cheesecloth, and stored at −80 °C until just prior to use with the Ames assay.

Smokeless tobacco products were extracted with DMSO according to the following procedure. The tobacco product was dispersed in DMSO (1:9, w/v) using an ultrasonic homogenizer. The dispersion was then incubated at 37 °C for 21 h. The dispersion was then centrifuged and filtered. The extract was stored at −80 °C prior to assay. Nicotine content of the DMSO extract was determined. While it can be envisaged that the bacterial content of smokeless tobacco products (Brotzge, 1984; Fisher and Hill, 1990; Rubinstein and Pedersen, 2002; Warke et al., 1999) might lead to the formation of artifacts from bacterial growth during the extraction process, DMSO has significant antimicrobial activity (Basch and Gadebusch, 1968).

The Ames assays were performed according to internationally accepted protocols (OECD, 1997). Only strains TA98 and TA100 were used and all assays were done with S9 activation with 20-min pre-incubation. The S9 (postmitochondrial supernatant in 0.154 M KCl) used for the Ames Assay was purchased from Molecular Toxicology Inc. (*a/k/a* Moltox Inc.). It came from the livers of male Sprague–Dawley rats induced with Aroclor 1254. Three replicate sets of assays were done for each extract. Mutagenic potency was estimated from the slope of the linear portion of the dose–response curve (Bernstein et al., 1982). The linear range used was 0–125 μg of TPM per plate in the case of smoking tobacco products. The linear range was 0–1389 μg of product per plate in the case of smokeless tobacco products. The corresponding nicotine concentrations were used to calculate slopes in terms of revertants per milligram nicotine. Thus the nicotine range used for the slope calculations was different for each different product.

For the smoking products, the dose–response curves obtained with TA98+S9 and TA100+S9 were very typical of those reported for tobacco smoke condensate. Condensates with a high relative proportion of nicotine gave lower slopes than those with lower proportions of nicotine. There was no significant dose–response when the DMSO extracts of the smokeless tobacco products were assayed with TA98+S9. The dose–response curves for the DMSO extracts of the smokeless tobacco products had shallow and somewhat variable slopes for the assays with TA100+S9. None of the dose–response data met the two-fold rule (Hamada et al., 1994; Cariello and Piegorsch, 1996), but gave overall positive slopes for the dose–response curves in the range of 0–1389 μg of product per plate. Therefore, the dose–response data were further evaluated with the SALM program (Kim and Margolin, 1999b). The SALM program was used with both the data from individual replicate extractions (e.g., three plates per dose level per replicate extract) and with the data from all replicates for a given set of extracts pooled (e.g., nine plates per dose level per sample). The SALM program provides a measure of relative potency and provides a statistical test for mutagenicity. The cited paper by Kim and Margolin and the references cited therein should be consulted for the statistical principles used by the SALM software.

3. Results

The results for the mainstream smoke chemical analyses are shown in Table 1. The results obtained are typical for the products analyzed. The TPM from the dark tobacco cigarette and from the CIM-7 flue-cured reference cigarette did not contain detectable glycerin or propylene glycol. This is consistent with what is known about the products. Likewise, the lack of propylene glycol in the TPM from the KY2R4F reference cigarette is consistent with the published formulation.

The results for the chemical analyses of the smokeless tobacco products are shown in Table 2. The two low-moisture snuff products contain lower percentages of nicotine than the percentage reported for the 1S2 reference dry snuff (NCSU, 2006). The presence of glycerin and menthol in both low-moisture products is also atypical of the 1S2. The two moist snuff products have nicotine concentrations typical of the 2S3 reference moist snuff (NCSU, 2006) and those reported for commercial products (Richter and Spierto, 2003).

Tables 3 and 4 give the results of the mutagenicity assays on TPM for TA98+S9 and TA100+S9, respectively. The data in Tables 3 and 4 are presented on a per milligram TPM basis. These data are presented so that readers may compare our results with others in the literature that are presented on a per milligram TPM basis. The results for the cigarette products are unremarkable and are well within the range of expected values for the types of cigarettes analyzed with the TPM generated under ISO conditions. The relative activity reported here of the TPM from the clove cigarette (kreteks) being less than that of the TPM from the KR2R4F confirms the findings in an unpublished INBIFO report (Roemer, 2002). Another unpublished INBIFO report covered the mutagenicity of the TPM from cigarillos (Gomm et al., 1996) and that study showed that the mutagenicity of cigarillo TPM was similar to that of the KY1R4F reference cigarette.

Tables 5 and 6 show the data for TA98+S9 and TA100+S9, respectively, when estimated per milligram of delivered nicotine for each of the smoking products. As expected, products with air-cured (burley, dark air-cured) tobaccos gave a higher number of revertants per milligram of nicotine than did the flue-cured products. This finding is believed due to the relatively higher amounts of nitrogen-containing, mutagenic pyrolysis products found in the TPM of air-cured tobaccos than found in flue-cured tobaccos.

One of the more interesting findings from this research was that under the conditions of the assays, the extracts of the smokeless tobacco products were not mutagenic with TA98+S9. Table 7 shows the results of the mutagenicity assays with TA100+S9 on the extracts of the smokeless

Table 1
Smoke analyses

Sample	Weight (mg/unit)		Puff count (per unit)		MS TPM (mg/unit)		CO (mg/unit)		Water (mg/unit)		Nicotine (mg/unit)		Tar (mg/unit)		Propylene glycol (mg/unit)		Glycerol (mg/unit)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Flue-cured cigarette	1112	10	10.7	0.5	24.6	1.8	15.5	0.4	4.24	0.45	1.49	0.12	18.9	1.2	BDL	BDL	2.12	0.13
Clove cigarette	789	12	7.45	0.19	11.0	0.0	6.80	0.2	0.76	0.02	0.74	0.01	9.51	0.05	0.151	0.007	0.834	0.043
Dark tobacco cigarette	969	6	6.54	0.13	12.6	0.6	14.6	0.6	1.00	0.22	0.45	0.01	11.2	0.4	NQ	NQ	NQ	NQ
CIM-7	972	5	7.99	0.21	14.6	0.4	12.4	0.3	1.53	0.09	1.03	0.02	12.0	0.4	BDL	BDL	BDL	BDL
KR2R4F	1076	5	8.79	0.25	10.9	0.3	12.1	0.5	0.80	0.04	0.75	0.02	9.33	0.26	NQ	NQ	0.947	0.027
Cigarillo 1	1075	33	11.6	0.4	49.2	3.3	52.3	1.7	8.32	0.85	2.28	0.09	38.6	2.4	0.310	0.025	0.400	0.013
Cigarillo 2	1077	9	12.0	0.7	58.2	3.8	52.7	2.9	12.0	1.3	2.7	0.02	43.5	2.5	BDL	BDL	BDL	BDL
Bidi 1	371	16	13.9	1.8	50.1	8.7	19.5	2.3	11.8	0.9	2.12	0.39	36.2	8.4	BDL	BDL	BDL	BDL
Bidi 2	459	8	16.4	2.5	43.2	12.7	18.1	3.0	11.3	4.1	2.23	0.41	29.6	8.2	BDL	BDL	BDL	BDL
Cigar 1	3415	199	35.8	1.1	229	32	NM	NM	64.4	12.2	9.52	0.34	155	20	2.16	0.20	BDL	BDL
Cigar 2	4309	51	50.5	9.3	215	11	NM	NM	63.6	9.8	6.28	0.06	146	1	1.93	0.14	BDL	BDL
Pipe tobacco 1	1501	1	80.3	25.0	150	21	NM	NM	52.5	2.8	5.34	0.77	91.8	17.7	4.92	0.45	0.734	0.22
Pipe tobacco 2	1501	1	82.9	49.8	198	25	NM	NM	73.5	10.7	6.27	1.03	118	15	1.30	0.36	15.4	3.2

Unit equals one cigarette, cigarillo, bidi, cigar, or portion of pipe tobacco.
Means and standard deviations presented are from three sample replicates.
BDL means below detection limit.
NM means not measured.
NQ means result obtained was below the limit of quantitation for the method.

Table 2
Smokeless tobacco analyses[a]

Sample	Country of origin	Menthol[b] (mg/g)		Nicotine[b] (mg/g)		Glycerol[b] (mg/g)		Dry matter (%)		Moisture (%)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Tableted low-moisture snuff	US	5.94	0.07	5.71	0.34	0.314	0.044	96.7	0.2	3.29	0.24
Low-moisture wintergreen snuff	US	6.44	0.15	9.17	0.06	0.561	0.025	95.7	0.2	4.34	0.23
Gutkha	India	7.60	0.18	2.10	0.11	1.97	0.18	92.7	0.3	7.34	0.30
Long-cut fruit-flavored moist snuff	US	BDL	BDL	14.3	0.1	BDL	BDL	48.5	0.1	51.5	0.1
Pouched moist snuff	US	BDL	BDL	13.3	0.4	BDL	BDL	49.4	0.2	50.6	0.2
Zarda chewing tobacco	India	21.8	0.5	26.7	1.0	46.3	1.0	87.5	0.0	12.5	0.0

BDL means below detection limit.
[a] All data reported on an "as is" basis; Means and standard deviations presented are from three sample replicates.
[b] The menthol, nicotine, and glycerol contents of the products were estimated from analysis of the DMSO extracts.

Table 3
Summary of TA98+S9 assays for smoking products, slope values in terms of revertants per milligram TPM

Sample	TPM dose range μg/plate	Replicate 1		Replicate 2		Replicate 3		All replicates	
		Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.
Flue-cured cigarette	0–125	1279	92	1151	62	1062	133	1164	82
Clove cigarette	0–125	1334	86	1264	56	1347	63	1315	58
Dark tobacco cigarette	0–125	2191	116	2641	133	2722	132	2518	87
CIM-7	0–125	1240	44	1040	24	1090	77	1123	45
KR2R4F	0–125	2299	114	2477	280	2213	92	2330	97
Cigarillo 1	0–125	2364	139	2588	176	2262	80	2405	83
Cigarillo 2	0–125	2407	153	2525	112	2593	82	2508	77
Bidi 1	0–125	904	39	990	12	940	54	945	29
Bidi 2	0–125	961	38	1039	68	1062	60	1021	45
Cigar 1	0–125	1585	35	1928	92	1816	58	1776	58
Cigar 2	0–125	1683	55	1888	71	2103	98	1892	76
Pipe tobacco 1	0–125	380	36	441	25	338	25	386	26
Pipe tobacco 2	0–100	283	25	315	49	209	49	269	32

Table 4
Summary of TA100+S9 assays for smoking products, slope values in terms of revertants per milligram TPM

Sample	TPM dose range μg/plate	Replicate 1		Replicate 2		Replicate 3		All Replicates	
		Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.
Flue-cured cigarette	0–125	635	37	666	18	678	27	660	27
Clove cigarette	0–125	555	47	611	18	519	32	562	55
Dark tobacco cigarette	0–125	770	37	525	43	674	101	656	54
CIM-7	0–125	543	29	502	36	568	26	538	26
KR2R4F	0–125	826	78	724	54	653	37	734	87
Cigarillo 1	0–125	653	35	728	29	568	14	650	23
Cigarillo 2	0–125	690	27	819	48	614	20	708	73
Bidi 1	0–125	482	28	469	23	500	35	484	31
Bidi 2	0–125	537	26	425	44	606	23	523	27
Cigar 1	0–125	659	27	589	15	522	43	590	21
Cigar 2	0–125	635	30	654	32	600	25	629	20
Pipe tobacco 1	0–125	337	31	371	23	475	33	394	21
Pipe tobacco 2	0–125	409	25	412	41	451	9	424	20

Table 5
Summary of TA98+S9 assays for smoking products, slope values in terms of revertants per milligram nicotine

Sample	Nicotine dose range μg/plate	Replicate 1		Replicate 2		Replicate 3		All replicates	
		Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.
Flue-cured cigarette	0–8	21,167	1516	19,055	1025	17,573	2198	19,265	1361
Clove cigarette	0–8	19,901	1281	18,856	831	20,100	935	19,619	861
Dark tobacco cigarette	0–4	60,938	3226	73,439	3705	75,696	3680	70,024	2417
CIM-7	0–9	17,590	623	14,758	344	15,471	1093	15,940	643
KR2R4F	0–9	33,237	1645	35,814	4055	31,992	1336	33,681	1404
Cigarillo 1	0–6	50,899	3003	55,722	3783	48,693	1727	51,771	1789
Cigarillo 2	0–6	51,644	3289	54,161	2403	55,624	1755	53,810	1652
Bidi 1	0–5	21,391	923	23,409	288	22,229	1285	22,343	690
Bidi 2	0–7	18,207	727	19,695	1297	20,135	1129	19,346	848
Cigar 1	0–5	37,526	825	45,663	2184	42,993	1365	42,061	1367
Cigar 2	0–4	57,729	1895	64,745	2431	72,126	3366	64,867	2594
Pipe tobacco 1	0–4	10,640	994	12,335	687	9446	693	10,807	723
Pipe tobacco 2	0–4	8945	796	9957	1564	6624	1537	8509	1007

tobacco products. This table shows the slopes of the linear portions of the dose–response curves and the associated error terms. The units for the slopes are revertants per milligram of product nicotine. Table 8 shows the results of using the SALM program on the mutagenicity data. Again, the results are presented on a nicotine-weight, not product-weight basis. Three values are provided for each replicate: (1) potency value, (2) fit p-value, and (3) mutagenicity p-value. The potency value is similar but not the same as the slope of the linear portion of the dose–response curve. Differences in the shape of the dose–response curve can result in potency values that differ from those estimated

Table 6
Summary of TA100+S9 assays for smoking products, slope values in terms of revertants per milligram nicotine

Sample	Nicotine dose range μg/plate	Replicate 1		Replicate 2		Replicate 3		All replicates	
		Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.
Flue-cured cigarette	0–8	10,505	606	11,028	299	11,230	445	10,921	453
Clove cigarette	0–8	8274	700	9115	270	7740	474	8376	815
Dark tobacco cigarette	0–4	21,400	1027	14,609	1198	18,741	2812	18,250	1488
CIM-7	0–9	7709	407	7130	514	8060	374	7633	365
KR2R4F	0–9	11,934	1123	10,464	779	9440	535	10,613	1252
Cigarillo 1	0–6	14,066	756	15,673	626	12,229	296	13,989	502
Cigarillo 2	0–6	14,809	575	17,580	1035	13,175	434	15,188	1563
Bidi 1	0–5	11,407	656	11,092	553	11,822	830	11,440	733
Bidi 2	0–7	10,172	491	8050	833	11,493	431	9905	503
Cigar 1	0–5	15,597	641	13,937	344	12,367	1009	13,967	507
Cigar 2	0–4	21,764	1041	22,431	1089	20,563	849	21,586	670
Pipe tobacco 1	0–4	9425	874	10,395	656	13,284	912	11,035	588
Pipe tobacco 2	0–4	12,935	793	13,031	1283	14,273	298	13,413	642

Table 7
Mutagenicity of extracts of smokeless tobacco products with TA100+S9, slope values in terms of revertants per milligram of nicotine

Sample	Nicotine dose range μg/plate	Country of origin	Replicate 1		Replicate 2		Replicate 3		All replicates	
			Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.	Slope	Std. Err.
Tableted low-moisture snuff	0–8	US	6270	990	4760	661	4202	560	5077	463
Low-moisture wintergreen snuff	0–13	US	1421	394	1369	705	2801	194	1863	354
Gutkha	0–3	India	13,902	985	4163	995	7577	1300	8547	1714
Long-cut fruit-flavored moist snuff	0–20	US	1120	146	1585	112	1182	151	1296	119
Pouched moist snuff	0–19	US	1615	170	1990	128	2265	172	1957	117
Zarda chewing tobacco	0–37	India	554	84	286	206	663	138	501	94

by other methods (Kim and Margolin, 1999b). The fit *p*-value describes the quality of the fit between the experimental data and the fitted model: the better the fit, the higher the *p*-value. The mutagenicity *p*-value describes the probability that the potency value is greater than zero. A mutagenicity *p*-value of less than 0.05 indicates mutagenicity. The major differences in results between the two methods for estimating mutagenic potency is that the SALM software indicated that two of the gutkha replicates and one of the low-moisture wintergreen snuff replicates had non-significant slopes while significant slopes were found with the other estimation procedure. An inspection of the dose–response curves showed that in those cases, the dose–response curves did not rise with increasing dose in the expected manner.

The dose–response curves for the extracts of the smokeless tobacco products with TA100+S9 are shown in Figs. 1–4. As noted earlier, none of the dose–response curves (see Figs. 1–4) showed a twofold increase in revertants per plate over background levels; and thus we used the SALM program to provide another estimate of mutagenic potency.

In Fig. 1, the dose–response curves for the two low-moisture snuff products are shown. The legend terms are as follows: (1) TLMS, tableted low-moisture snuff; (2) LMWGS, low-moisture wintergreen snuff. R1, R2 and R3 are the identifiers for the replicate samples. Except for a single step-change in the dose–response curve o Replicate 1 of the extract of the LMWGS, there is no monotonically rising increase in response with increase in dose. This could be the reason the SALM program reported no significant mutagenic potency for that replicate. The dose–response curves for the moist snuff products are shown in Fig. 2. LCFFMS, long-cut fruit-flavored moist snuff; and PMS, pouched moist snuff. These two products appeared to give the most consistent dose–response curves of the products included in this study. This consistency may stem from the fact that both products have high consumer acceptance and are believed to be made in large factories.

Fig. 3 shows the dose–response curves for the Guthka extracts with TA100+S9. Replicate 1 showed significant mutagenicity while the other two replicates did not show significant mutagenicity with the SALM program although slopes could be calculated from the linear parts of the dose–response curves. The dose–response curves for the extracts of Zarda with TA100+S9 are shown in Fig. 4. These curves indicate much more cytotoxicity than did the other samples. This increased cytotoxicity may have been due to the additives to the tobacco.

It is important to note that the SALM software uses all points in the dose–response curve unless the internal error-checking routines delete them as likely errors (Kim and Margolin, 1999b). While use of the twofold rule could result in the conclusion that none of the snuff extracts were mutagenic, the results from the SALM program show that all the samples were mutagenic with TA100+S9.

Table 8
mutagenicity of extracts of smokeless tobacco products with TA100+S9 estimated with SALM program, potency values in terms of revertants per milligram of nicotine

Sample	Nicotine dose range (μg/plate)	Replicate 1			Replicate 2			Replicate 3			All replicates		
		Potency value	Fit *p*-value	Mutagenicity *p*-value	Potency value	Fit *p*-value	Mutagenicity *p*-value	Potency value	Fit *p*-value	Mutagenicity *p*-value	Potency value	Fit *p*-value	Mutagenicity *p*-value
Tableted low-moisture snuff	0–32	11,887	0.147	0.000	9156	0.329	0.000	9729	0.767	0.000	10,275	0.026	0.000
Low-moisture wintergreen snuff	0–51	200	0.192	0.084	2953	0.376	0.000	6981	0.989	0.000	5415	0.783	0.000
Gutkha	0–12	30,049	0.946	0.000	NS	0.332	0.500	31	0.10	0.480	20,579	0.030	0.000
Long-cut fruit-flavored moist snuff	0–80	514	0.526	0.000	3698	0.987	0.000	366	0.156	0.000	2876	0.964	0.000
Pouched moist snuff	0–74	3533	0.486	0.000	4000	0.775	0.000	3893	0.818	0.000	3778	0.188	0.000
Zarda chewing tobacco	0–149	1056	0.961	0.000	1163	0.176	0.000	1159	0.747	0.000	1130	0.840	0.000

Table 9 shows the ratios of the mutagenic potencies for TA98+S9 and TA100+S9 for the TPM from smoking products and pipe tobaccos. Two items of note: (1) products made with air-cured tobaccos gave higher values for the TA98+S9/TA100+S9 ratio than did all flue-cured products; and (2) the two tobacco blends smoked in pipes had values of that ratio that were less than unity. However, that latter finding may be the result of the use of pipes as opposed to the tobacco blend as pipes when compared with tubular smoking articles (e.g., cigarettes, cigars) have been shown to give reduced biological activity with the same blend (Billimoria, 1975). Similar ratios have been noted by other researchers (Sato et al., 1977; Cerna and Angelis, 1985).

4. Discussion

Mutagenicity generally is not an acceptable attribute for a product intended for human consumption. However, some foods and beverages (e.g., broiled meat and coffee) show some evidence of mutagenic activity (Sugimura, 2000). It may be argued that nutrition is essential for life and some foods need to be cooked to minimize the levels of pathogens and toxins, and mutagens arising from such cooking may not present an unreasonable risk of disease. On the other hand, items consumed for pleasure, such as tobacco, preferably should not be mutagenic. Conventional tobacco smoking products produce mutagenic smoke as shown by the experimental data in this article and references cited herein. Even electrically heated cigarettes produce mutagenic TPM albeit at much lower potency than conventional products (Tewes et al., 2003). Therefore, there has been an increased focus on converting smokers who will not quit to users of smokeless tobacco products (Levy et al., 2004). However, not all smokeless tobacco products present the same risk to the user (Rodu and Jansson, 2004). Furthermore, much of the literature on health risks of smokeless tobacco products has focused on TSNAs and NNK, in particular (Nilsson, 2006; Rodu and Jansson, 2004). In addition, the concentrations of TSNAs in smokeless tobacco products are reportedly below the limit of detection for the Ames assay (Grasso et al., 1996). Furthermore, endogenous polyphenolic compounds have been shown to be antimutagenic towards NNK (Miller et al., 1996). Thus, mutagenicity observed is likely caused by yet unidentified compounds that are mutagenic with TA100+S9, but not TA98+S9. For example, some aldehydes and dicarbonyl compounds that have been reported in tobacco products are active with TA100+S9 but not TA98+S9 (Dillon et al., 1998; Aeschbacher et al., 1989). Pyrolysis products from plant materials and carbohydrates have been reported to be mutagenic with TA100+S9 but not TA98+S9 (Derache, 1982; Kitts et al., 2006). Certain Maillard reaction products, which may be created during the curing of tobacco and in the manufacture of tobacco products, could result in mutage-

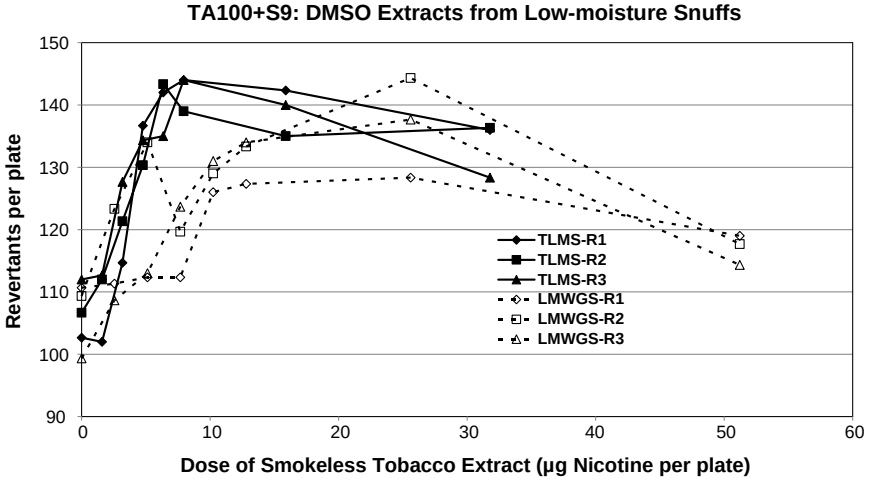

Fig. 1. Dose–response curve for TA100+S9: DMSO extracts from low-moisture snuffs.

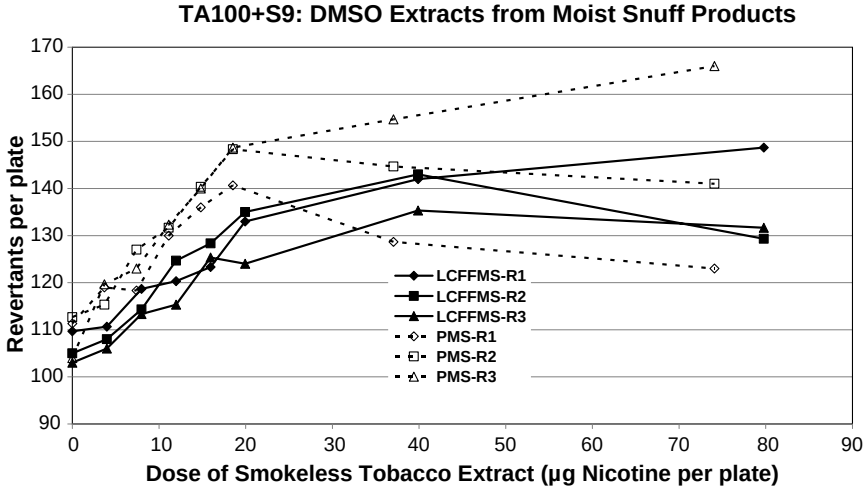

Fig. 2. Dose–response curve for TA100+S9: DMSO extracts from moist snuff products.

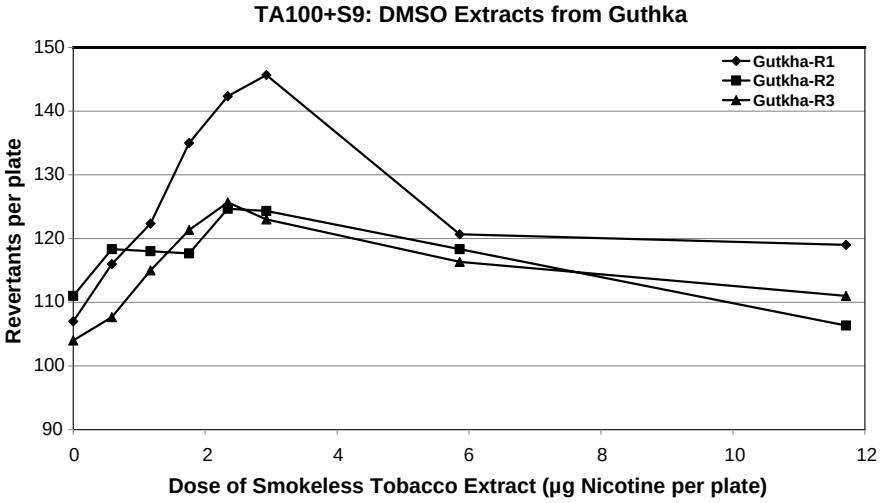

Fig. 3. Dose–response curve for TA100+S9: DMSO extracts from guthka.

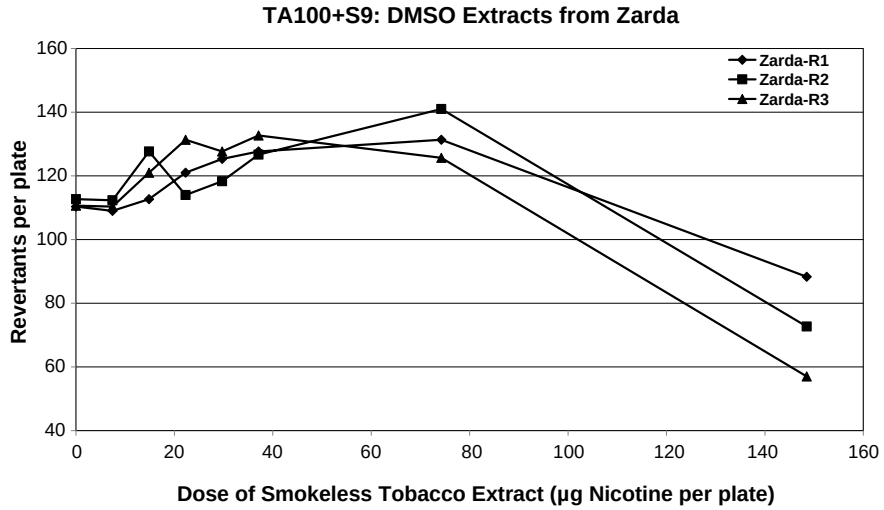

Fig. 4. Dose–response curve for TA100+S9: DMSO extracts from zarda.

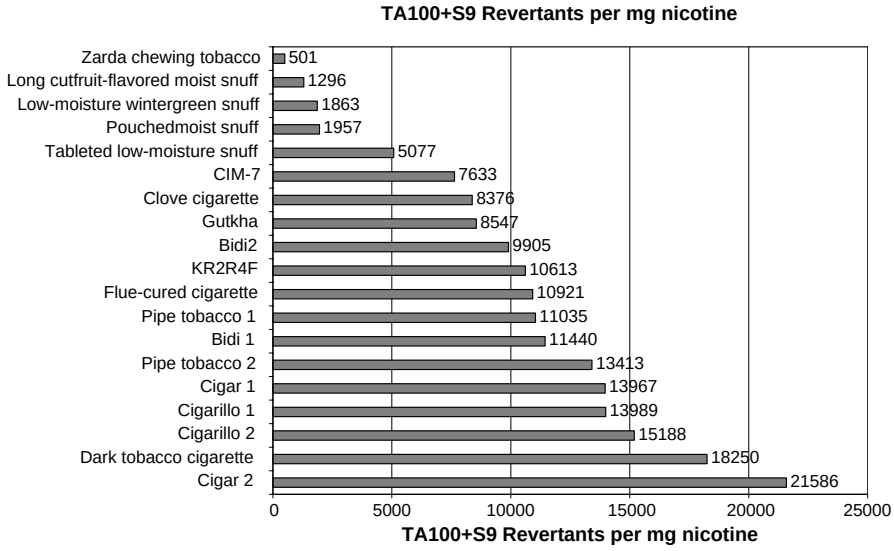

Fig. 5. Comparison of TA100+S9 Revertants per milligram nicotine for various types of tobacco products.

nicity with TA100+S9 but not TA98+S9 (Powrie et al., 1981; Pool et al., 1984).

Our work, along with the work of others as cited, already has shown that most western-style smokeless tobacco products are not mutagenic with TA98+S9. Numerous studies as cited herein have shown that the TPM from conventional cigarettes is mutagenic with TA98+S9. Furthermore, the epidemiology of cigarettes with higher TPM mutagenicity with TA98+S9 shows more evidence of smoking-related diseases than cigarettes with lower TPM mutagenicity under the same condition (Lee, 2001; Malaveille et al., 1989; Curvall et al., 1987). Thus, this provides additional evidence for the use of some smokeless tobacco products in place of cigarettes for those who chose not to refrain from tobacco use (Levy et al., 2004; Nilsson, 2006).

We do not know if the compounds in TPM after activation with S9 that are active with TA100 are the same as those in smokeless tobacco samples that are active with TA100 after the S9 metabolic activation. We do not know if revertants caused by TPM represent more or less toxicity than do revertants caused by smokeless tobacco. However, in order to estimate the possible differences in doses of mutagens received from use of smoking products with those received from use of smokeless tobacco products, we have calculated mutagenic activity on the basis of nicotine delivery for smoking products and nicotine content for smokeless products.

We are not alone in the use of adjusting measure of potential harm to nicotine delivery and a similar approach was just used by Laugesen and Fowles to compare the toxicities of mainstream cigarette smoke from cigarettes with differing nicotine deliveries (Laugesen and Fowles, 2006). We realize that this approach to comparing mutagenicities of products may be subject to criticism because of differences in intake, uptake, and metabolism among users of

Table 9
Ratios of TA98+S9/TA100+S9 for smoking products

Sample	TA98+S9 revertants per milligram Nicotine	TA100+S9 revertants per milligram Nicotine	Ratio TA98+S9/ TA100+S9
Flue-cured cigarette	19,265	10,921	1.764
Clove cigarette	19,619	8376	2.342
Dark tobacco cigarette	70,024	18,250	3.837
CIM-7	15,940	7633	2.088
KR2R4F	33,681	10,613	3.174
Cigarillo 1	51,771	13,989	3.701
Cigarillo 2	53,810	15,188	3.543
Bidi 1	22,343	11,440	1.953
Bidi 2	19,346	9905	1.953
Cigar 1	42,061	13,967	3.011
Cigar 2	64,867	21,586	3.005
Pipe tobacco 1	10,807	11,035	0.979
Pipe tobacco 2	8509	13,413	0.634

such products, and such differences are noted in many journal articles and governmental reports that are too numerous to cite. Our results for all samples assayed with TA100+S9 are shown graphically in Fig. 5. These results also are reflective of current thinking that use of some smokeless tobacco products is likely to present less risk of cancer than use of cigarettes and other smoking products (Nilsson, 2006; Rodu and Jansson, 2004).

5. Conclusions

Our initial objective for this work was to characterize the mutagenic response for a range of smoked and smokeless tobacco products. We found, although it is far from a perfect solution, that we could compare the mutagenic potency of mainstream smoke condensate from smoking articles with that of smokeless tobacco products if we based our comparisons on mutagenic potency expressed on a nicotine basis. Thus, we were able to compare the mutagenicity of TPM, measured in terms of revertants per milligram nicotine with the mutagenicity of extracts of smokeless tobacco products measured in term of revertants per milligram of nicotine in the product. This approach showed that some of the smokeless products assayed would result in less mutagenicity transmitted to the user than would occur with smoking products. Furthermore, we were not able to detect significant mutagenicity when the extracts of the smokeless tobacco products were tested with TA98+S9. This provides support to a current public health discussion that it may be better for smokers who cannot stop their need for nicotine to switch to smokeless tobacco products.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yrtph.2007.05.003.

References

Aeschbacher, H.U., Wolleb, U., Loliger, J., Spadone, J.C., Liardon, R., 1989. Contribution of coffee aroma constituents to the mutagenicity of coffee. Food Chem. Toxicol. 27, 227–232.

Anczak, J.D., Nogler 2nd, R.A., 2003. Tobacco cessation in primary care: maximizing intervention strategies. Clin. Med. Res. 1, 201–216.

Basch, H., Gadebusch, H.H., 1968. In vitro antimicrobial activity of dimethylsulfoxide. Appl. Microbiol. 16, 1953–1954.

Bates, C., Fagerstrom, K., Jarvis, M.J., Kunze, M., McNeill, A., Ramstrom, L., 2003. European Union policy on smokeless tobacco: a statement in favour of evidence based regulation for public health. Tob. Control 12, 360–367.

Bernstein, L., Kaldor, J., McCann, J., Pike, M.C., 1982. An empirical approach to the statistical analysis of mutagenesis data from the *Salmonella* test. Mutat. Res. 97, 267–281.

Billimoria, H., 1975. The reducing property of tobacco smoke. 2. The influence of smoking vehicle with particular reference to pipe. Beiträge zur Tabakforschung 8, 193–198.

Brown, B., Avalos, J., Lee, C., Doolittle, D., 2001. The effect of tobacco smoke, nicotine, and cotinine on the mutagenicity of 4-(methylnitrosamino.-1-(3-pyridyl.-1-butanol) NNAL. Mutat. Res. 494, 21–29.

Brotzge, K., 1984. Microbial examination of pipe, snuff, & chewing tobacco products Fall/Winter, 1983. Brown & Williamson Report, June 22, 1984. <http://legacy.library.ucsf.edu/tid/zcj41f00/> (accessed 31.01.07.).

Cariello, N.F., Piegorsch, W.W., 1996. The Ames test: the two-fold rule revisited. Mutat. Res. 369, 23–31.

CDC, 2000. The Surgeon General's report on reducing tobacco use: Warning Label Fact Sheet. <http://www.cdc.gov/tobacco/sgr/sgr_2000/warninglabel.pdf/> (accessed 31.01.07.).

Cerna, M., Angelis, K.J., 1985. Mutagenicity in the *Salmonella*/microsome assay of tobacco condensates formed during pipe smoking. Mutat. Res. 143, 161–164.

Curvall, M., Romert, L., Norlen, E., Enzell, C.R., 1987. Mutagen levels in urine from snuff users, cigarette smokers, and non tobacco users-a comparison. Mutat. Res. 188, 105–110.

Deaton, A.P., 1987. Evaluation of tobacco extracts in the *Salmonella* mutagenicity test. Lorillard Report, October 19, 1987. <http://legacy.library.ucsf.edu/tid/mkd21e00/> (accessed 31.01.07.).

DeMarini, D.M., 2004. Genotoxicity of tobacco smoke and tobacco smoke condensate: a review. Mutat. Res. 567, 447–474.

Derache, R., 1982. Foods pyrolysis and risk of toxicity [French]. Cah. Nutr. Diet. 17, 38–45.

Dillon, D., Combes, R., Zeiger, E., 1998. The effectiveness of *Salmonella* strains TA100, TA102, and TA104 for detecting mutagenicity of some aldehydes and peroxides. Mutagenesis 13, 19–26.

Field, T., Rickert, W.S., Kaiserman, M.J., 2001. Mainstream and sidestream yields of "tar", nicotine and CO from various types of cigars when smoked under a variety of conditions. Tob. Sci. Res. Conf. 55, 44 (abstract 42).

Fisher, P.R., Hill, J.A., 1990. Microbial examination of pipe, snuff, and chewing tobacco products Spring/Summer 1990. Brown & Williamson Report, August 7, 1990. <http://legacy.library.ucsf.edu/tid/hzt03f00/> (accessed 31.01.07.).

Gomm, W., Meisgen, T., Reininghaus, W., Tewes, F., Voncken, P., von Holt, K., 1996. Report P 0500/3256 in vitro mutagenicity of total particulate matter of the test cigarillos Kentucky Tobaccos, West Tobaccos, and Maria 47P in the *Salmonella typhimurium* reverse mutation assay. INBIFO report, November 22, 1996. <http://legacy.library.ucsf.edu/tid/eyv67d00/> (accessed 31.01.07.).

Grasso, P., Benford, D., Mann, A.H., 1996. Assessment of experimental evidence relating to smokeless tobacco and oral cancer. Report No. RI93/TOX001, Robens Institute of Industrial and Environmental Health and Safety. <http://legacy.library.ucsf.edu/tid/aoh42c00/> (accessed 31.01.07.).

Guttenplan, J.B., 1987. Mutagenic activity in smokeless tobacco products sold in the USA. Carcinogenesis 8, 741–743.

Hamada, C., Wada, T., Sakamoto, Y., 1994. Statistical characterization of negative control data in the Ames *Salmonella*/microsome test. Environ. Health Perspect. 102 (Suppl. 1), 115–119.

Hatsukami, D.K., Lemmonds, C., Tomar, S.L., 2004. Smokeless tobacco use: harm reduction or induction approach? Prev. Med. 38, 309–317.

ISO, 2000. Cigarettes—determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine, ISO 4387:2000, International Organization for Standardization, Geneva, Switzerland.

ISO, 1999. Tobacco and tobacco products—atmosphere for conditioning and testing. ISO 3402:1999. ISO 4387:2000, International Organization for Standardization, Geneva, Switzerland.

Jansson, T., Romert, L., Magnusson, J., Jenssen, D., 1991. Genotoxicity testing of extracts of a Swedish moist oral snuff. Mutat. Res. 261, 101–115.

Joza, P., Rickert, W.S., Kaiserman, M.J., 2001. A comparison of yields of "tar", nicotine and CO from pipe tobacco determined under various smoking conditions. 55th Tobacco Science Research Conference, Program Booklet and Abstracts, 55, #43, 45.

Kim, B.S., Margolin, B.H., 1999a. Prediction of rodent carcinogenicity utilizing a battery of in vitro and in vivo genotoxicity tests. Environ. Mol. Mutagen. 34, 297–304.

Kim, B.S., Margolin, B.H., 1999b. Statistical methods for the Ames *Salmonella* assay: a review. Mutat. Res. 436, 113–122.

Kitts, D.D., Wu, C.H., Kopec, A., Nagasawa, T., 2006. Chemistry and genotoxicity of caramelized sucrose. Mol. Nutr. Food Res. 50, 1180–1190.

Laugesen, M., Fowles, J., 2006. Marlboro ultrasmooth: a potentially reduced exposure cigarette? Tob. Control 15, 430–435.

Lee, P.N., 2001. Lung cancer and type of cigarette smoked. Inhal. Toxicol. 13, 951–976.

Levy, D.T., Mumford, E.A., Cummings, K.M., Gilpin, E.A., Giovino, G., Hyland, A., Sweanor, D., Warner, K.E., 2004. The relative risks of a low-nitrosamine smokeless tobacco product compared with smoking cigarettes: estimates of a panel of experts. Cancer Epidemiol. Biomarkers Prev. 13, 2035–2042.

Malaveille, C., Vineis, P., Esteve, J., Ohshima, H., Brun, G., Hautefeuille, A., Gallet, P., Ronco, G., Terracini, B., Bartsch, H., 1989. Levels of mutagens in the urine of smokers of black and blond tobacco correlate with their risk of bladder cancer. Carcinogenesis 10, 577–586.

Massey, E.D., 2002. Tobacco smoke: an in vitro genotoxic perspective. Rec. Adv. Tob. Sci. 28, 69–103.

Miller, C., Castonguay, A., Teel, R.W., 1996. Modulation of the mutagenicity and metabolism of the tobacco-specific nitrosamine 4(-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by phenolic compounds. Mutat. Res. 386, 221–233.

Mortelmans, K., Zeiger, E., 2000. The Ames *Salmonella*/microsome mutagenicity assay. Mutat. Res. 455, 29–60.

Nair, U., Bartsch, H., Nair, J., 2004. Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: a review of agents and causative mechanisms. Mutagenesis 19, 251–262.

NCSU, 2006. The Reference Smokeless Tobacco Products and analytical data on them are available from the Tobacco Analytical Lab, Crop Science Dept., Box 8604, North Carolina State University, Raleigh, NC 27695-8604, USA.

Nilsson, R., 2006. De minimus non curat lex—virtual thresholds for cancer initiation by tobacco specific nitrosamines—prospects for harm reduction by smokeless tobacco. Int. J. Occup. Med. Environ. Health 19, 6–35.

Nilsson, R., 1998. A qualitative and quantitative risk assessment of snuff dipping. Regul. Toxicol. Pharmacol. 28, 1–16.

Niphadkar, M.P., Bagwe, A.N., Bhisey, R.A., 1996. Mutagenic potential of Indian tobacco products. Mutagenesis 11, 151–154.

OECD, 1997. Guideline 471 (updated). Bacterial reverse mutation test. In: Guidelines for testing of chemicals. Organization for Economic Co-operation and Development, OECD Publishing, Paris, France.

Pool, B.L., Roper, H., Roper, S., Romruen, K., 1984. Mutagenicity studies on *N*-nitrosated products of the Maillard browning reaction: *N*-nitroso-fructose-amino acids. Food Chem. Toxicol. 22, 797–801.

Powrie, W.D., Wu, C.H., Rosin, M.P., Stich, H.P., 1981. Clastogenic and mutagenic activities of Maillard reaction model systems. J. Food Sci. 46, 1433–1438, and 1445.

Richter, P., Spierto, F.W., 2003. Surveillance of smokeless tobacco nicotine, pH, moisture, and unprotonated nicotine content. Nicotine Tob. Res. 5, 885–889.

Rickert, W.S., Trivedi, A., Wright, W., 2002. Effect of smoking condition and method of collection on TA98 and TA100 response to crude smoke condensate (CSC) from control cigarettes (Kentucky Reference 1R4F, 1R5F and a Canadian flue-cured monitor). CORESTA Congress, New Orleans, 2002, Smoke Science/Product Technology Groups (abstract ST7).

Rodu, B., Godshall, W.T., 2006. Tobacco harm reduction: an alternative cessation strategy for inveterate smokers. Harm Reduct. J. 3, 37–50.

Rodu, B., Jansson, C., 2004. Smokeless tobacco and oral cancer: a review of the risks and determinants. Crit. Rev. Oral Biol. Med. 15, 252–263.

Roemer, E., 2002. Chemical composition, in vitro cytotoxicity, and in vitro mutagenicity of two kretek brands. Executive summary of INBIFO report, September 2, 2002. <http://legacy.library.ucsf.edu/tid/dag77c00/> (accessed 31.01.07.); also see Draft tables and figures P 0268/2200 in vitro mutagenicity of total particulate matter in mainstream smoke from 2 kretek market cigarettes and from the reference cigarette 2R4F *Salmonella typhimurium* reverse mutation assay Project Kretek (PT). Part of draft INBIFO report, August 26, 2002. <http://legacy.library.ucsf.edu/tid/bag77c00/> (accessed 31.01.07.).

Roemer, E., Stabbert, R., Rustemeier, K., Veltel, D.J., Meisgen, T.J., Reininghaus, W., Carchman, R.A., Gaworski, C.L., Podraza, K.F., 2004. Chemical composition, cytotoxicity and mutagenicity of smoke from US commercial and reference cigarettes smoked under two sets of machine smoking conditions. Toxicology 195, 31–52.

Rubinstein, I., Pedersen, G.W., 2002. Bacillus species are present in chewing tobacco sold in the United States and evoke plasma exudation from the oral mucosa. Clin. Diagn. Lab. Immunol. 9, 1057–1060.

Sato, S., Seino, S., Ohka, T., Yahagi, T., Nagao, N., Matsushima, T., Sugimura, T., 1977. Mutagenicity of smoke condensates from cigarettes, cigars, and pipe tobacco. Cancer Lett. 3, 1–8.

Stamm, S.C., Zhong, B.Z., Whong, W.Z., Ong, T., 1994. Mutagenicity of coal-dust and smokeless-tobacco extracts in *Salmonella typhimurium* strains with differing levels of *O*-acetyltransferase activities. Mutat. Res. 321, 253–264.

Sugimura, T., 2000. Nutrition and dietary carcinogens. Carcinogenesis 21, 387–395.

Surgeon General, 1986. The health consequences of using smokeless tobacco. A report of the Advisory Committee to the Surgeon General. U.S. Department of Health and Human Services, Public Health Service, Bethesda, MD, NIH Publication 86-2874.

Tewes, F.J., Meisgen, T.J., Veltel, D.J., Roemer, E., Patskan, G., 2003. Toxicological evaluation of an electrically heated cigarette. Part 3: genotoxicity and cytotoxicity of mainstream smoke. J. Appl. Toxicol. 23, 341–348.

Wahlberg, I., Ringberger, T., 1999. Smokeless tobacco. In: Davis, D.L., Nielsen, M.T. (Eds.), Tobacco Production, Chemistry, and Technology. Blackwell Science, Malden, MA, pp. 452–460.

Warke, R.G., Kamat, A.S., Kamat, M.Y., 1999. Irradiation of chewable tobacco mixes for improvement in microbiological quality. J. Food Prot. 62, 678–681.

Wehlburg, A.F., 1999. Cigars and cigarillos. In: Davis, D.L., Nielsen, M.T. (Eds.), Tobacco Production, Chemistry, and Technology. Blackwell Science, Malden, MA, pp. 440–451.

Health Risks Associated With Cigar Smoking

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This article summarizes principal findings from a conference convened by the American Cancer Society in June 1998 to examine the health risks of cigar smoking. State-of-the-science reports were presented and 120 attendees (representing government and private agencies, academia, health educators, and tobacco control experts) participated in panels and summary development discussions. The following conclusions were reached by consensus: (1) rates of cigar smoking are rising among both adults and adolescents; (2) smoking cigars instead of cigarettes does not reduce the risk of nicotine addiction; (3) as the number of cigars smoked and the amount of smoke inhaled increases, the risk of death related to cigar smoking approaches that of cigarette smoking; (4) cigar smoke contains higher concentrations of toxic and carcinogenic compounds than cigarettes and is a major source of fine-particle and carbon monoxide indoor air pollution; and (5) cigar smoking is known to cause cancers of the lung and upper aerodigestive tract.

JAMA. 2000;284:735-740 www.jama.com

CIGAR SMOKING HAS INCREASED rapidly in recent years, coincident with the aggressive glamorization and promotion of cigars.[1(pp195-219)] The American Cancer Society convened a conference June 15 and 16, 1998, in Washington, DC, to review current knowledge of the health risks of cigar smoking. The 120 invited attendees represented governmental and private agencies, academia, health educators, and tobacco control experts. Tobacco control experts with a specific interest in cigar smoking were invited to present papers. Many of these speakers had review articles published in the recent National Cancer Institute monograph on cigar smoking[1] and were asked to provide an update on their research efforts. In addition, a series of panels discussed the implications of the data presented at the conference, and a final panel of all conference attendees provided a forum for summary discussion. This article summarizes the data presented at the conference and the formal discussions at the concluding session.

How Do Cigars Differ From Cigarettes?

Cigars are defined by the US Department of the Treasury as "any roll of tobacco wrapped in leaf tobacco or in any substance containing tobacco"; cigarettes are defined as a "roll of tobacco wrapped in paper or a substance not containing tobacco."[2] There is no universal agreement on how to classify the many types of cigars available today (TABLE 1).

A fundamental difference between cigar and cigarette tobacco is in the processing. Cigars consist of filler (the inner part of the cigar), a binder, and a wrapper, all of which are made with air-cured and fermented tobaccos. US cigarettes contain a blend of heat-cured and air-cured tobaccos as major components and a small percentage of sun-cured (oriental) tobaccos; they do not contain fermented tobacco.

Air-curing tobacco involves hanging the whole tobacco plant or individually primed leaves (if intended for cigar use) in barns or sheds for 30 to 40 days. In heat-curing, leaves of tobacco are hung on tiers in barns where the air is gradually warmed to a temperature of 70°C to 75°C over a period of 5 to 7 days. After curing, the leaves are typically aged for 2 or more years. Fermentation entails packing the tobacco leaves with placement in fermentation rooms for 3 to 5 weeks; they are subsequently removed, repacked, and returned to the fermentation rooms several times to achieve the desired flavor and aroma.

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Table 1. Cigar Types and Characteristics*

Classification System	Cigar			
	Weight, g	Length, mm	Diameter, mm	Description
US Department of the Treasury[2]†				
Small	≤1.36	. . .	. . .	. . .
Large	>1.36	. . .	. . .	. . .
US Federal Trade Commission[3]†				
Little	<1.36	. . .	. . .	. . .
Medium	1.36-4.54	. . .	. . .	. . .
Large	>4.54	. . .	. . .	. . .
Hoffman and Hoffman,[1(pp55-104)] 1998				
Little	0.9-1.3	70-100	. . .	Shaped like cigarettes; some with filter tips
Small	1.3-2.5	70-120	. . .	Also known as cigarillos, cheroots; usually have wood or plastic mouthpieces
Regular	5-17	110-150	≤17	Usually rolled to a tip on at least 1 end and banded; machine made or hand rolled
Premium	≤22	127-214	12-23	Almost all are hand rolled; currently cost from US $4 to >$20

*Ellipses indicate none specified.
†Converted from the US Customary System of measurement to the International System for comparability.

Cigar tobacco compared with US cigarette tobacco is rich in nitrate (1.4%-2.1% vs 0.1%-1.7%). During fermentation, which contributes greatly to the flavor and aroma of cigar tobacco, nitrate is partially reduced to the strong N-nitrosating nitrite, which reacts with amines to form nitrosamines. Cigar tobacco, compared with cigarette tobacco, is rich in the highly carcinogenic N′-nitrosonornicotine (NNN) (3.0-4.5 µg/g vs 1.8-3.0 µg/g) and in 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (1.2-4.5 µg/g vs 0.1-1.0 µg/g); these tobacco-specific carcinogens are formed from nornicotine and nicotine. During fermentation, cigar tobacco is greatly reduced in protein, reducing sugars (0.9%-2.7%), phytosterols (0.14%-0.16%), and polyphenols (<0.1%), but in cigarette tobacco, sugar levels range from 5.5% to 20%, phytosterol levels range from 0.3% to 4.5%, and polyphenol levels range from 3.0% to 5.0%. Consequently, cigar smoke is rich in nitrogen oxides (150-300 µg/g of tobacco burned vs 90-150 µg/g from cigarettes), ammonia, and nitrosamines. Furthermore, cigar smoke tends to have a higher pH than cigarette smoke, which increases the amount of free nicotine in the particulate and vapor phases of the smoke.[1]

Trends in Cigar Smoking

Between the years 1993 and 1997, the consumption of all types of cigars in the United States increased by 46.4%, reversing a steady decline (66%) in cigar consumption from 1964 to 1993.[1(pp21-53)] Between 1993 and 1997, consumption of large cigars and cigarillos increased 69.4%.[4]

Premium cigars accounted for only a small part of this increased consumption. The vast majority of cigar smokers smoke other less expensive large cigars; small cigars, known as cigarillos; and little cigars, which resemble cigarettes and are packaged similarly but have a wrapper that contains tobacco (Table 1).[1(55-104)]

Data on cigar sales are readily available from the US Department of Agriculture, but prevalence data on current cigar smoking are sparse. Since cigar-smoking rates had remained low for many years, questions on cigar use were omitted from many national health surveys. Surveys among California adults between 1990 and 1996 showed that the increases in cigar smoking occurred primarily among younger more educated adults.[5] Some data indicate that adult men are more likely to smoke cigars than adult women and that cigar smoking is increasing among adolescents in both sexes, surpassing the use of smokeless tobacco.[6] Data from the 1997 Youth Risk Behavior Survey[7] indicated that 31% of male adolescents had smoked at least 1 cigar in the past month and that cigar smoking prevalence among adolescent girls was nearly 11%. Data from the 1998 National Household Survey on Drug Abuse indicate that the rate of current cigar use among those aged 12 years or older increased from 5.9% in 1997 to 6.9% in 1998, a statistically significant increase, and that an estimated 5.6% of youths aged 12 to 17 years were current cigar users in 1998. Statistically significant increases in past-month-cigar use were also reported for (1) white, non-Hispanic males, (2) those living in the Northeast, (3) those with some college education, and (4) the unemployed.[8]

An additional concern relates to initial evidence suggesting that some adolescent cigar smokers may engage in a practice known as *blunting*, whereby the cigar filler tobacco is removed and replaced with marijuana and possibly other illicit drugs.[9]

Marketing and Promotion of Cigars

Beginning in the mid 1980s, the cigar industry intensified its public relations efforts in the United States through strategies such as cigar dinners, product placement in movies, feature stories, sporting events, and the development of cigar-friendly lifestyle magazines (such as *Cigar Aficianado*). Electronic and print media report America's "rediscovery" of the premium cigar smoked by the affluent and successful members of society.[1(pp195-219),10,11] The sale of cigars has expanded from tobacco stores,

upscale restaurants, and luxury hotels to availability at gas stations, grocery stores, liquor stores, variety stores, and menswear sections in department stores. These promotional efforts resemble those undertaken in the early stages of the smokeless tobacco campaign, which ultimately became a major health problem.[12,13] Advertising and promotional activities for cigars, similar to those for cigarettes, routinely include sexual imagery, affluence, and celebrity endorsement (explicitly and implicitly). Unlike cigarette marketing promotions, those for cigars are not required to mention the potential health risks associated with tobacco use, which gives the impression that cigars are a "safe" product.

The Public Perceptions of Cigars

National data indicate that 46.6% of cigar smokers surveyed believe that cigar smoking is a high-risk behavior for developing cancer.[14] However, they evidence an "optimistic bias" in their estimate of their own risk of developing cancer in the next 20 years: only 8.7% consider themselves to be at high risk.[14-16] Compared with nonsmokers, cigar smokers also underestimate the cancer risk of exposure to environmental cigar smoke.[14]

The glamorized image of cigar smokers presented in the media appears to be accepted both by those who smoke cigars and those who do not. A large fraction of both groups (about 40%) perceive cigar smokers as relatively well-to-do, well-educated, older managers or executives. Cigar smokers are more likely to associate athleticism with cigar smoking than are nonsmokers, which may be due in part to media imagery of sports figures smoking cigars at a victory celebration.[14]

Pharmacology and Abuse Potential of Cigars

Whether cigars deliver nicotine at a level capable of producing dependence is a function of the degree of cigar smoke inhalation, the rate of nicotine absorption, the development of tolerance to nicotine, the age of initiation, and the duration of exposure. The amount of nicotine in a cigar is approximately proportional to the amount of tobacco it contains; this may range from less than 1 g to more than 20 g of tobacco, depending on the cigar size and the amount of tobacco incorporated in its components.[17] Thus, the nicotine in the smoke of a single cigar can vary from an amount approximate to that in the smoke of a single cigarette to the amount generated by smoking a pack or more of cigarettes. Cigars are capable of providing high levels of nicotine at a sufficiently rapid rate to produce clear physiological and psychological effects that lead to dependence, even if the smoke is not inhaled.

The manner in which tobacco products are smoked and their ability to deliver nicotine is influenced by the pH of the smoke. Accurate measurement of smoke pH has eluded scientists, and measurements obtained vary depending on the method used. However, if the concept of smoke pH is defined as the pH of the smoke and aerosol particles, it is generally correct to assume that cigar smoke aerosol particles are less acidic relative to cigarette smoke aerosol particles. Furthermore, the alkalinity of cigar smoke aerosol particles relative to cigarette smoke aerosol particles tends to deter inhalation, although cigar smoke is often partially inhaled, especially by current and former cigarette smokers.[1(pp181-193),7,18] Studies indicate that two thirds of those who smoke both cigars and cigarettes (>40% of cigar smokers) inhale cigar smoke, compared with less than 15% of cigar smokers who never smoked cigarettes.[1(pp181-193),19]

Definitive studies of nicotine tolerance and withdrawal have not been conducted on cigar smokers. Some research suggests that cigars produce fewer abstinence-induced withdrawal symptoms than cigarettes, but their nicotine delivery characteristics and the daily patterns of cigar smoking by many persons suggest a distinct potential to produce dependence.[1(pp181-193)] The number of cigar smokers in the population who smoke infrequently, who consume few cigars per day, and who inhale minimally suggests that cigar use beginning in adulthood may be less likely to induce dependence than that resulting from cigarette smoking.

Chemistry and Toxicology

Most of what is known about the nature and chemistry of tobacco and tobacco smoke is derived from studies on cigarettes, with little work specifically focused on cigar smoke. Tobacco and tobacco smoke contain about 6700 compounds, of which about 4000 have been identified in tobacco smoke.[20] At least 63 of these compounds are known to be carcinogenic, including 11 known human carcinogens.[21] The chemistry of cigar smoke is believed to be qualitatively similar to that of cigarettes, except for differences caused by the aging and fermentation of cigar tobacco and by the use of additives (primarily in cigarettes). Quantitative differences are primarily due to differences in the smoke pH and lower oxygen concentrations (resulting from the poor porosity of the tobacco wrappers compared with the paper wrappers of cigarettes).

A class of highly carcinogenic compounds known as tobacco-specific, N-nitrosamines (TSNA) is present in cigar smoke at significantly higher levels than in cigarette smoke.[1(pp55-104)] Examination on a "per gram of tobacco smoked" basis reveals that *tar*, defined as the total particulate matter collected by a Cambridge filter after subtracting moisture and nicotine; carbon monoxide; and ammonia are produced in greater quantities by cigars than cigarettes. When equal doses are applied, the tar produced by cigars exerts greater tumorigenic activity in mice compared with the tar from cigarettes, because cigar tar contains higher concentrations of carcinogenic polycyclic aromatic hydrocarbons.[1(pp55-104)22-24]

Environmental Tobacco Smoke

Sidestream smoke (the aerosol emitted from the burning cone of a cigar, cigarette, or pipe during the interval be-

tween puffs and the portion of the inhaled smoke that is not retained and is exhaled[25]) contributes significant pollutants to the environment in the form of carbon monoxide, nitrogen oxides, respirable suspended particulate matter, nicotine, polycyclic aromatic hydrocarbons, and other compounds, and sidestream smoke from cigars does so to a greater degree than the sidestream smoke of cigarettes, when equal amounts of tobacco are burned.[1(pp55-104,161-179)] Compared with a single cigarette (0.55 g) smoked to 70% of its mass, a large cigar smoked 70% emits about 20 times the carbon monoxide, 5 times the respirable particles, and twice the amount of polycyclic aromatic hydrocarbon.[1(pp161-179)]

One study of environmental pollutants from tobacco smoke found the levels of carbon monoxide at cigar banquets and in some cigar smokers' homes equal to carbon monoxide concentrations on crowded California freeways. The indoor carbon monoxide level measured at a cigar banquet averaged 10 ppm over the 3-hour-20-minute event, and peak levels were comparable to that in a busy parking garage. By comparison, the ambient outdoor carbon monoxide level at rush hour was 1 to 2 ppm.[1(pp161-179),26] The Environmental Protection Agency's standard for carbon monoxide places the maximum permissible level at an average of 9 ppm over an 8-hour period.[27]

Mathematical models designed for the analysis and interpretation of indoor air pollution measurements suggest that typical levels of respirable tar particles from cigar smoking in homes, offices, and restaurants may exceed the National Ambient Air Quality Standard for outdoor fine-particle air pollution (65 $\mu g/m^3$ on a 24-hour average).[1(161-179),27] Thus, it is clear that cigar smoke can be a major source of indoor air pollution.

Cigar Smoking and Cancer Risk

Since the 1950s, epidemiologic studies of cigar smokers have found increased risk of oral, esophageal, laryngeal, and lung cancer.[1(105-158),25,28-31] The risks of cancers of the oral cavity and esophagus are similar among cigarette and cigar smokers, probably due to the similar doses of tobacco smoke delivered directly to these areas by cigars and cigarettes.[1(pp105-158)] Lung cancer risk is less strongly associated with cigar smoking than with cigarette smoking, but risk increases with the number of cigars smoked per day and depth of inhalation. Men who smoke 3 or more cigars per day and report moderate inhalation experience lung cancer death at about two thirds the rate of men who smoke 1 pack of cigarettes a day.[1(pp105-158)] A recent case-control study from Europe (where inhalation patterns and tobacco composition in cigars may differ from those in the United States) found a relative risk (RR) of 9.0 (95% confidence interval [CI], 5.8-14.1) for lung cancer among European cigar and cigarillo smokers,[32] substantially higher than the lung cancer risk in older studies of US cigar smokers.[1]

Additional estimates of the risk of cancer in cigar smokers come from an analysis of data from the Cancer Prevention Study 1 (CPS-1) of the American Cancer Society, a cohort study conducted between 1959 and 1972.[1(pp105-158)] Of the 442 455 white male subjects in CPS-I, 15 191 were primary cigar smokers and had never smoked cigarettes, 7404 were secondary cigar smokers and had previously smoked cigarettes, 10 300 were mixed smokers and currently smoking both cigars and cigarettes; and 175 000 were cigarette-only smokers. The cancer risks for these groups were compared with rates for 92 300 men who never smoked based on mortality information. The analysis included consideration of a dose-response effect for all groups related to numbers of cigars smoked per day and degree of self-reported smoke inhalation.

This study provides strong support for an increased risk in cigar smokers for cancers of the lung, esophagus, larynx, oral cavity, and, probably, pancreas. The increase in risk appears to be roughly proportional to the degree of exposure to the cigar smoke. For example, the death rate from cancers of the oral cavity among male cigar smokers, compared with lifelong nonsmokers, is nearly 8 times higher (RR, 7.92; 95% CI, 5.12-11.69); similarly, the death rate from cancer of the larynx is about 10-fold higher (RR, 10.02; 95% CI, 4.0-20.6). For both of these cancers, a dose-response effect is evident and is related to the frequency of cigars smoked.[1(pp105-158)] The death rate from esophageal cancer is 3 to 4 times higher in male cigar smokers than in lifelong male nonsmokers (RR, 3.60; 95% CI, 2.2-5.6). The increase in cancer risk associated with cigar smoking is thus greater in the oropharynx and larynx than in the more distant esophagus. The mucosa of the esophagus is exposed only to tobacco carcinogens that have been dissolved in saliva and swallowed but not to the smoke itself. Similarly, lung cancer risk is higher among cigar smokers who report inhaling the smoke than in those who report not inhaling, and higher among cigar smokers who previously smoked cigarettes than among those who only smoked cigars.[1(pp105-158)]

Other Health Effects of Cigar Smoking

Several older studies suggested that cigar smoking increases the risk for coronary heart disease (CHD), chronic obstructive pulmonary disease, and aortic aneurysm, particularly among heavy cigar smokers (≥3 cigars a day) and those who inhale smoke deeply,[31] but there was no clear consensus that cigar smoking causes CHD. The 1983 Surgeon General's Report, which mainly emphasized the hazards of cigarette smoking, concluded that those who smoke only cigars did not appear to experience substantially greater risks than nonsmokers.[33] The report notes that the category of nonsmokers also includes passive smokers so that the control group contains persons exposed to environmental tobacco smoke. However, an analysis of CPS-1 data concluded that "cigar smokers who smoke several cigars per day or who inhale [the smoke] are at increased risk for CHD."[1(pp105-158)]

A second large cohort study, the Cancer Prevention Study II (CPS-II), was

Table 2. Implication for Patient Counseling

- Ask patients if they smoke cigars and advise them of the associated health risks.
- Advise patients that smoking cigars is not a safe alternative to cigarette smoking.
- Inform patients that cigar smoke contains a number of carcinogenic and noxious substances and poses risks to the smoker and persons environmentally exposed.
- Caution adolescents of the double dangers of *blunting*, removing the cigar filler tobacco and replacing it with marijuana or other drugs, exposing them to the drug as well as nicotine and carcinogens in the wrapper.

initiated by the American Cancer Society in 1982. A recent analysis of these data examined death rates due to CHD in relation to cigar smoking.[34] After excluding men who had ever smoked pipes or cigarettes regularly, approximately 7000 current cigar smokers, 7000 former cigar smokers, and 113 000 men who had never regularly smoked tobacco remained in the analysis. As with cigarette smoking, the association between cigar smoking and death due to CHD was strongest among younger men and current rather than former smokers. There was no apparent increase in risk for cigar smokers aged 75 years or older or among former cigar smokers. Among men younger than 75 years, current cigar smokers experienced a death rate from CHD about one third higher than those who never smoked. This relationship held over the range of cigars smoked per day and was not limited to men who reported inhaling cigar smoke (although unintentional inhalation obviously occurs).

Policy Issues

Fewer federal and state regulations pertain to cigars than to cigarettes or smokeless tobacco.[35] Cigars are not included in many of the federal and state policies involving health warnings on tobacco, prohibition of sales to minors, and taxation. However, a recent Federal Trade Commission report to Congress recommended health warnings on all labeling and advertising for cigar products; prohibition on electronic advertising such as radio and television for all tobacco products, including all sizes and types of cigars; and consistency in regulating youth access to tobacco products, including cigars.[3]

Evidence of the health hazards and an alarming increase in rates of cigar smoking underscore the pressing need for cigars to be included in a coherent national policy on tobacco use and dependence. The research on the heavy impact of secondhand cigar smoke on indoor air pollution is particularly relevant for restricting smoking in restaurants and other public places. Although smoking is usually considered an adult problem, tobacco use by children and adolescents is of a particular concern. In addition to research showing high levels of adolescent cigar use, evidence is emerging that young persons use cigars to mask illicit substance abuse.[9] The serious health risks associated with tobacco use, including cigars, highlights the need for a broad and inclusive national policy that addresses the constellation of tobacco products and their use by all age groups.

Conclusions

The available scientific knowledge on the health risks of cigar smoking is more than sufficient to conclude that cigar smoking is a cause of cancer and a serious risk to the public health. The increase in cigar smoking has particular implications for both research and policy development. First, rates of cigar smoking are increasing, and not just among adults. Both male and female adolescents are using cigars, and their rates of use have met or exceeded those of adults before 1993. Second, similar to other tobacco products, cigars contain nicotine, which is highly addictive; smoking cigars instead of cigarettes does not reduce the risk of becoming addicted to nicotine. Third, as the number of cigars smoked and the amount of smoke inhaled increases, the risks of death related to cigar smoking approach those of cigarette smokers. Switching to cigars from cigarettes does not necessarily reduce the risk of death from a tobacco-caused illness. Fourth, cigar smoking does not just affect cigar smokers: environmental cigar smoke contains high concentrations of toxic and carcinogenic compounds and can be a major contributor to indoor air pollution, in amounts greater than that produced from cigarettes. Most importantly, cigar smoking is known to cause cancer of the lung and upper aerodigestive tract.

The weight of the evidence indicates that smoking cigars is not a safe alternative to cigarette smoking. The recent increase in rates of cigar smoking and its risks to health underscore the pressing need for a comprehensive national tobacco policy and for active patient educational efforts (TABLE 2). Laws and regulations limiting the marketing of cigarettes and access to cigarettes by minors should be applied to all tobacco products.

A number of avenues for research to define further the health risks exists. Such research could include efforts to understand better the nature of tobacco addiction associated with cigar smoking; the identification of biomarkers of the uptake of carcinogens, carbon monoxide, and nicotine in active cigar smokers; and the relationship of atmospheric nicotine to body fluid cotinine in nonsmokers exposed to environmental cigar smoke. Research is necessary to establish clearly the risks of cigar smoking associated with CHD, cancers, and pulmonary disease. Future studies should focus on morbidity in susceptible groups, including younger cigar smokers; give attention to the type, size, and pattern of use of cigars; examine intermediate markers of morbidity and mortality; and address the temporal relationships between cigar smoking and the development of disease.

Acknowledgment: We thank Monica Cobis, BA, for her assistance in organizing the Cigar Smoking Health Risks: State-of-the-Science conference, held June 15-16, 1998, in Washington, DC.

REFERENCES

1. National Cancer Institute. *Cigars: Health Effects and Trends*. Bethesda, Md: US Dept of Health and Human Services, Public Health Service. Smoking and Tobacco Control Monograph No. 9. NIH publication 98-4302. Available at: http://rex.nci.gov/NCI_MONOGRAPHS/MONO9HTM. Accessibility verified July 7, 2000.
2. IRC §5702 (1986).
3. Federal Trade Commission. Report to Congress: cigar sales and advertising and promotional expendi-

tures for calendar years 1996 and 1997. July 1999. Available at: http://www.ftc.gov/os/1999/9907/cigarreport1999.htm. Accessed August 8, 1999.
4. US Department of Agriculture. *Tobacco Situation and Outlook Report*. Washington, DC: US Dept of Agriculture, Commodity Economics Division, Economic Research Service; 1998. Publication TBS-241.
5. Pierce JP, Gilpin EA, Farkas AJ, et al. *Tobacco Control in California: Who's Winning the War? An Evaluation of the Tobacco Control Program, 1989-1996*. La Jolla: University of California, San Diego; 1998.
6. Centers for Disease Control and Prevention. Tobacco use among high school students. *MMWR Morb Mortal Wkly Rep*. 1998;47:229-233.
7. Centers for Disease Control and Prevention. Cigar smoking among teenagers—United States, Massachusetts, and New York, 1996. *JAMA*. 1997;278:17-19.
8. US Department of Health and Human Services, Substance Abuse and Mental Health Services Administration, Office of Applied Studies. *Summary of Findings from the 1998 National Household Survey on Drug Abuse*. Rockville, Md: US Dept of Health and Human Services; 1999.
9. US Dept of Health and Human Services, Office of Inspector General. *Youth Use of Cigars: Patterns of Use and Perceptions of Risk*. Washington, DC: US Dept of Health and Human Services, Office of Inspector General; February 1999.
10. Hamilton K. Blowing smoke. *Newsweek*. July 21, 1997:54-56.
11. Trillin C. All puffed up. *Time*. July 15, 1996:18.
12. Altman DG, Jackson C. Adolescent tobacco use and the social context. In: Shumaker SA, Schron EB, Ockene JK, McBee WL, eds. *The Handbook of Health Behavior Change*. 2nd ed. New York, NY: Springer Publishing Co; 1998:305-329.
13. US Dept of Health and Human Services. *The Health Consequences of Using Smokeless Tobacco: A Report of the Advisory Committee to the Surgeon General*. Washington, DC: US Dept of Health and Human Services; 1986. NIH publication 86-2874.
14. Baker F, Dye JT, Ainsworth SR, Denniston M. Risk perception and cigar smoking behavior. Presented at: American Cancer Society's Cigar Smoking Health Risks: State-of-the-Science Conference; June 15, 1998; Washington, DC.
15. Strecher VJ, Kreuter MW, Kobrin SC. Do cigarette smokers have unrealistic perceptions of their heart attack, cancer, and stroke risks? *J Behav Med*. 1995;18:45-54.
16. Weinstein ND. Unrealistic optimism about susceptibility to health problems: conclusions from a community-wide sample. *J Behav Med*. 1987;10:481-500.
17. Henningfield JE, Fant RV, Radvius A, Frost S. Nicotine concentration, smoke pH, and whole tobacco aqueous pH of some cigar brands and types popular in the United States. *Nicotine Tobacco Res*. 1999;1:163-181.
18. Wald NJ, Watt HC. Prospective study of effect of switching from cigarettes to pipes or cigars on mortality from three smoking-related diseases. *BMJ*. 1997;314:1860-1863.
19. Gilpin EA, Pierce JP. Cigar smoking in California: 1990-1996. *Am J Prev Med*. 1999;16:195-201.
20. Green CR, Rodgman A. The tobacco chemists' research conference: a half-century forum for advances in analytical methodology of tobacco and its products. *Recent Advan Tobacco Sci*. 1996;22:131-304.
21. Hoffmann D, Hoffmann I, Wynder EL. The changing cigarette, 1950-1997: facts and expectations: report of Canada's Expert Committee on Cigarette Toxicity Reduction. In: Conference Proceedings and Invited Papers; September 20-22, 1998; Toronto, Ontario. Paper No. 2.
22. Croninger AB, Graham EA, Wynder EL. Experimental production of carcinoma with tobacco products, V: carcinoma induction in mice with cigar, pipe, and all-tobacco cigarette tar. *Cancer Res*. 1958;18:1263-1271.
23. Kensler CJ. The pharmacology of tobacco smoke: effect of chronic exposure. In: James G, Rosenthal T, eds. *Tobacco and Health*. Springfield, Ill: Charles C Thomas Publishers; 1962:5-20.
24. Homburger F, Treger A, Baker JR. Mouse skin painting with smoke condensates made of pipe, cigar, and cigarette tobaccos. *J Natl Cancer Inst*. 1963;31:1445-1459.
25. US Department of Health and Human Services. *Smoking and Health: Other Forms of Tobacco Use*. Washington, DC: US Dept of Health and Human Services; 1979. DHHS publication PHS 79-500066.
26. Klepeis NE, Ott WR, Repace JL. The effect of cigar smoking on indoor levels of carbon monoxide and particles. *J Expo Anal Environ Epidemiol*. 1999;9:622-635.
27. Environmental Protection Agency, Office of Air and Radiation Clean Air Act Web site. Available at: http://www.epa.gov/airs/criteria.html. Accessed May 31, 2000.
28. Wynder EL, Graham EA. Tobacco smoking as a possible etiologic factor in bronchiogenic carcinoma: a study of six hundred and eighty-four approved cases. *JAMA*. 1950;143:329-336.
29. Levin ML, Goldstein H, Gerhardt PR. Cancer and tobacco smoking: a preliminary report. *JAMA*. 1950;143:336-338.
30. Zaridze D, Peto R. Tobacco: a major international health hazard. In: Proceedings of an International Meeting Organized by the International Agency for Research on Cancer and Co-Sponsored by the All-Union Cancer Research Centre of the Academy of Medical Sciences of the USSR; June 4-16, 1986. IARC 74.
31. Iribarren C, Tekawa IS, Sidney S, Friedman GD. Effect of cigar smoking on the risk of cardiovascular disease, chronic obstructive pulmonary disease, and cancer in men. *N Engl J Med*. 1999;340:1773-1780.
32. Boffetta P, Pershagen G, Joeckel KH, et al. Cigar and pipe smoking and lung cancer risk: a multicenter study from Europe. *J Natl Cancer Inst*. 1999;91:697-701.
33. US Surgeon General. *The Health Consequences of Smoking: Cardiovascular Disease*. Rockville, Md: US Dept of Health and Human Services, Public Health Service Office on Smoking and Health; 1983.
34. Jacobs EJ, Thun MJ, Apicella LF. Cigar smoking and death from coronary heart disease in a prospective study of United States men. *Arch Intern Med*. 1999;159:2413-2418.
35. Centers for Disease Control and Prevention. State tobacco control highlights, 1996. Atlanta, Ga: Centers for Disease Control and Prevention; 1996. CDC publication 099-4895.

What smells so? Has somebody been burning a Rag, or is there a Dead Mule in the Back yard? No, the Man is Smoking a Five-Cent Cigar.
—Eugene Field (1850-1895)

Prospective study of effect of switching from cigarettes to pipes or cigars on mortality from three smoking related diseases

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BMJ 1997;314:1860–3

Abstract

Objective: To estimate the extent to which cigarette smokers who switch to cigars or pipes alter their risk of dying of three smoking related diseases—lung cancer, ischaemic heart disease, and chronic obstructive lung disease.

Design: A prospective study of 21 520 men aged 35-64 years when recruited in 1975-82 with detailed history of smoking and measurement of carboxyhaemoglobin.

Main outcome measures: Notification of deaths (to 1993) classified by cause.

Results: Pipe and cigar smokers who had switched from cigarettes over 20 years before entry to the study smoked less tobacco than cigarette smokers (8.1 g/day *v* 20 g/day), but they had the same consumption as pipe and cigar smokers who had never smoked cigarettes (8.1 g) and had higher carboxyhaemoglobin saturations (1.2% *v* 1.0%, $P < 0.001$), indicating that they inhaled tobacco smoke to a greater extent. They had a 51% higher risk of dying of the three smoking related diseases than pipe or cigar smokers who had never smoked cigarettes (relative risk 1.51; 95% confidence interval 0.96 to 2.38), a 68% higher risk than lifelong non-smokers (1.68; 1.16 to 2.45), a 57% higher risk than former cigarette smokers who gave up smoking over 20 years before entry (1.57; 1.04 to 2.38), and a 46% lower risk than continuing cigarette smokers (0.54; 0.38 to 0.77).

Conclusion: Cigarette smokers who have difficulty in giving up smoking altogether are better off changing to cigars or pipes than continuing to smoke cigarettes. Much of the effect is due to the reduction in the quantity of tobacco smoked, and some is due to inhaling less. Men who switch do not, however, achieve the lower risk of pipe and cigar smokers who have never smoked cigarettes. All pipe and cigar smokers have a greater risk of lung cancer than lifelong non-smokers or former smokers.

Introduction

It is recognised that cigarette smokers inhale tobacco smoke while men who smoke only pipes or cigars tend not to. Pipe and cigar smokers are at lower risk of the main smoking related diseases, probably in part because of this difference in inhaling habit[1] and because they may smoke less tobacco. There is evidence that smokers who switch from cigarettes to pipes or cigars tend to maintain their acquired inhaling habits.[2-7] The extent to which this occurs and the extent to which it negates the potential health benefits associated with smoking cigars or pipes rather than cigarettes is uncertain. We used data from the British United Provident Association (BUPA) study to examine these points in greater detail in relation to three diseases caused by smoking—namely, lung cancer, ischaemic heart disease, and chronic obstructive lung disease. In particular, we assessed the merits of switching from smoking cigarettes to smoking pipes and cigars.

Subjects and methods

The BUPA study, a prospective study of 21 520 professional and business men aged 35-64 years who attended the BUPA Medical Centre in London for a routine health examination between 1975 and 1982, has been described previously.[8] At the time of each examination, which included a blood pressure measurement, a detailed history of smoking was obtained, including self reported inhaling habits classified into four categories (nil, slight, moderate, deep). A blood sample was collected, carboxyhaemoglobin saturation and various other factors (including serum cholesterol) measured, and serum samples stored at −40°C. The study was restricted to men with NHS numbers so that their NHS records could be flagged and the Office of Population Censuses and Surveys could inform us of all deaths and their certified causes. Further information was then sought from the doctor who certified death. Eight hundred and twenty nine men were lost to follow up because they emigrated. The average follow up time was 14 years and 4 months. This report is based on the deaths that occurred in the study up to October 1993. The codes of ICD-9 (international classification of diseases, ninth revision) used to classify the three specified smoking related diseases were 162 for lung cancer, 410-414 for ischaemic heart disease, and 416, 491, 492, 496, and 519 for chronic obstructive lung disease.

To assess the benefits of switching from cigarettes to cigars or pipes, or both, we compared mortality from the three smoking related diseases in current pipe or cigar smokers who had switched (switchers) from smoking cigarettes at least 20 years before entry to the study (that is, 20 years before 1975-82) with that in pipe or cigar smokers who were current smokers at entry and had never smoked cigarettes (non-switchers). The 20 year interval between switching and the date of the examination was selected to avoid the excess health hazards attributable to past cigarette smoking. We verified this by showing that men who had stopped smoking cigarettes for at least 20 years before entry to the study and had not switched to pipes or cigars had death rates from the three specified diseases that were similar to those in lifelong non-smokers. We also compared death rates in the switchers with those in cigarette smokers who did not smoke cigars or pipes, with those in former smokers, and with those in lifelong non-smokers.

Consumption of tobacco was estimated on the basis of one cigarette containing 1 g of tobacco, one small cigar (cheroot) containing 2 g, and one large cigar containing 5 g. The weight of tobacco used by pipe smokers

was recorded directly on the questionnaire. Carboxyhaemoglobin saturations were measured (taking the mean of two measurements) with an IL182 co-oximeter, as described in detail previously, for all men at the time they attended the medical centre.[9] Risks of mortality from the three specified diseases were compared by using Cox's proportional hazards survival analysis adjusted for age at entry to the study. We used survival analysis to take account of the differing lengths of follow up, which were mainly attributable to the eight year recruitment period. The results were also analysed by logistic regression (results not shown), which gave virtually identical results. The association between carboxyhaemoglobin measurements and self described inhaling category was examined by analysis of variance.

Results

Table 1 shows the data collected in 1975-82 on median tobacco consumption and carboxyhaemoglobin saturations according to smoking group: men who currently smoked cigars or pipes and had never smoked cigarettes, men who currently smoked cigars and pipes but smoked cigarettes at least 20 years previously, and men who smoked only cigarettes. Tobacco consumption was similar in the two groups of pipe and cigar smokers (switchers and non-switchers) (table 1), but the median carboxyhaemoglobin saturation was higher in the switchers (1.2% *v* 1.0%, $P<0.0001$ by the Wilcoxon rank sum test). The mean carboxyhaemoglobin saturations in both groups of pipe and cigar smokers were much lower than in current cigarette smokers (table 1). In lifelong non-smokers and in former smokers the median carboxyhaemoglobin saturation (reflecting endogenous production and exposure to atmospheric carbon monoxide) was 0.7% (10th-90th centile 0.4-1.1).

Table 2 shows self described inhaling category according to smoking group. One third of the switchers, and about half of the non-switchers said that they inhaled, and 95% of current cigarette smokers said that they inhaled; this indicates clear differences between the three groups.

Table 2 also shows an increasing trend in carboxyhaemoglobin saturation with deeper self described inhaling; cigarette smokers had higher saturations than other smokers except for men in the lightest category of smoking. This was also the case after we allowed for type of product smoked and weight of tobacco smoked ($P<0.0001$ by analysis of variance). In the pipe and cigar smokers there was no material difference in carboxyhaemoglobin saturations within each inhaling category between switchers and non-switchers; self described inhaling therefore accounted for the difference in carboxyhaemoglobin saturations shown in table 1.

Table 3 shows the mortality and the number of deaths by 1993 from the three smoking related diseases and from all causes for the three groups of current smokers and for former smokers relative to lifelong non-smokers. The combined risk of the three diseases in current cigarette smokers was 3.18 times higher (95% confidence interval 2.55 to 3.84) than in lifelong non-smokers; individual relative risk estimates were 2.27 for ischaemic heart disease, 16.4 for lung cancer, and 29.5 for chronic obstructive lung disease. Having given up smoking cigarettes 20 or more years before entry to the study reduced the risk to about that of a lifelong non-smoker (rows 1 and 2 in

Table 1 Numbers of pipes, cigars, and cigarettes smoked each day, weight of tobacco smoked each day, and carboxyhaemoglobin saturations according to smoking group. Figures are medians (10th-90th centiles)

Smoking group	No of men	No of pipes, cigars, and cigarettes	Weight of tobacco (g)	Observed carboxyhaemoglobin saturation (%)
Pipe/cigar smoker, never smoked cigarettes (non-switchers):				
Pipes only	472	6 (2-16)	12.1 (4.0-20.3)	1.4 (0.7-3.6)
Cigars only	651	2 (0.4-6)	4.7 (2.0-17.8)	0.9 (0.5-2.4)
Both	186	4 (2-12)	11.5 (5.5-24.9)	1.0 (0.5-2.6)
Either or both	1309	3 (0.9-11)	8.1 (2.1-20.3)	1.0 (0.6-3.2)
Pipe/cigar smoker, switched from cigarettes over 20 years ago (switchers):				
Pipes only	187	7 (3-16)	12.1 (4.0-20.3)	1.9 (0.7-4.8)
Cigars only	272	2 (0.6-6)	5.0 (2.0-17.4)	1.0 (0.6-4.2)
Both	63	5 (2-13)	10.8 (6.0-22.4)	1.2 (0.6-3.6)
Either or both	522	4 (1-12)	8.1 (2.0-20.3)	1.2 (0.6-4.5)
Current cigarette smokers (cigarettes/day):				
1-14	947	8 (3-12)	8.0 (3.0-12.0)	1.8 (0.8-4.6)
15-24	1387	20 (15-20)	20.0 (15.0-20.0)	4.4 (2.0-7.0)
25-34	1133	30 (25-30)	30.0 (25.0-30.0)	5.7 (3.2-8.7)
≥35	717	40 (35-50)	40.0 (35.0-50.0)	6.2 (3.6-9.6)
All amounts	4184	20 (7-40)	20.0 (7.0-40.0)	4.6 (1.4-8.0)

Table 2 Self described inhaling status and median carboxyhaemoglobin saturations by smoking group and inhaling category

	Self described inhaling category (% men in each smoking group)				Median carboxyhaemoglobin saturation by inhaling category (%)			
Smoking group	Nil	Slight	Moderate	Deep	Nil	Slight	Moderate	Deep
Pipe/cigar smoker, never smoked cigarettes (non-switchers):								
Pipes only	61	23	13	3	1.2	2.0	2.8	3.9
Cigars only	69	20	9	2	0.8	0.9	2.0	3.6
Both	52	22	21	5	1.1	1.8	3.2	2.2
Either or both	67	21	10	2	0.9	1.3	2.6	3.7
Pipe/cigar smoker, switched from cigarettes over 20 years ago (switchers):								
Pipes only	40	33	23	5	1.3	2.0	2.6	4.7
Cigars only	55	26	14	5	0.9	1.0	2.3	1.4
Both	74	17	8	1	1.0	2.1	2.4	4.3
Either or both	49	28	18	5	0.9	1.4	2.6	3.8
Currently cigarette smoker (cigarettes/day):								
1-14	7	23	55	15	1.3	1.5	2.0	2.1
15-24	5	11	61	23	3.7	3.7	4.4	4.9
25-34	3	8	56	32	5.2	5.2	5.7	5.8
≥35	4	9	42	44	5.0	5.9	6.2	6.4
All amounts	5	13	55	27	3.2	3.4	4.6	5.4

Table 3 Relative mortality (with 95% confidence intervals) according to smoking group for the three specified diseases compared with mortality among lifelong non-smokers

Smoking group at entry	Total No of men	Ischaemic heart disease		Lung cancer		Chronic obstructive lung disease		All three diseases		All cause mortality	
		Relative mortality	No of deaths	Relative mortality	No of deaths	Relative mortality	No of deaths	Relative mortality	No of deaths	Relative mortality	No of deaths
Lifelong non-smoker	6539	1.00	125	1.00	7	1.00	1	1.00	133	1.00	346
Former cigarette smoker, stopped smoking over 20 years before entry	1465	1.05 (0.77 to 1.45)	59	1.01 (0.26 to 3.91)	3	†	1	1.07 (0.79 to 1.45)	63	1.11 (0.92 to 1.34)	162
Pipe/cigar smoker, never smoked cigarettes (non-switchers)	1309	0.98 (0.67 to 1.44)	33	3.19* (1.07 to 9.50)	6	†	1	1.11 (0.78 to 1.59)	40	1.23 (0.99 to 1.75)	113
Pipe/cigar smoker, switched from cigarettes over 20 years before entry (switchers)	522	1.29 (0.88 to 1.99)	25	8.64* (3.19 to 23.3)	9	†	1	1.68* (1.16 to 2.45)	35	1.33 (1.03 to 1.73)	69
Current cigarette smoker	4182	2.27* (1.81 to 2.84)	193	16.4* (7.55 to 44.2)	77	29.5* (3.96 to 220)	20	3.13* (2.55 to 3.84)	290	2.26 (1.97 to 2.58)	540

*P<0.05 compared with mortality in lifelong non-smokers.
†Too few deaths to give reliable estimate.

Table 4 Mortality from three specified diseases (ischaemic heart disease, lung cancer, and chronic obstructive lung disease) in men who switched from smoking cigarettes to smoking pipes or cigars, or both, 20 or more years before entry to study, relative to mortality in four reference groups

Reference group*	Mortality (95% CI) relative to reference group
Lifelong non-smokers	1.68 (1.16 to 2.45)
Former cigarette smokers, stopped smoking over 20 years ago (switchers)	1.57 (1.04 to 2.38)
Pipe/cigar smokers, never smoked cigarettes (non-switchers)	1.51 (0.96 to 2.38)
Current cigarette smokers	0.54 (0.38 to 0.77)

*Risk in reference group set at 1.00. Relative mortality estimate can be derived by selecting entry in column in table 3 headed "All three diseases" relating to pipe/cigar smoker, switched from cigarettes over 20 years before entry (switchers) and dividing this by entry for smoking group taken to be reference group.

table 3). The risk in switchers was much less than among continuing cigarette smokers (rows 4 and 5, $P<0.001$) but somewhat higher than the risk in lifelong pipe and cigar smokers (rows 3 and 4, $P=0.07$). Mortality from all causes was also graded as expected. The rates were lower in non-smokers; successively higher in former smokers, pipe and cigar smokers who had never smoked cigarettes, and pipe and cigar smokers who had previously smoked cigarettes; and substantially higher in cigarette smokers. The relative risks of ischaemic heart disease were not materially changed after we allowed for serum cholesterol concentration and blood pressure. They were 0.97 (95% confidence interval 0.71 to 1.33) for former smokers, 1.00 (0.68 to 1.47) for pipe and cigar smokers who never smoked cigarettes, 1.31 (0.85 to 2.03) for those who had switched from cigarettes, and 2.18 (1.74 to 2.73) for cigarette smokers.

Table 4 shows the risk of the three smoking related diseases in the switchers compared with other groups. The risk of all three diseases combined was 46% lower in switchers than in continuing cigarette smokers (relative risk 0.54) and 68% higher than in lifelong non-smokers. It was 51% higher than in pipe or cigar smokers who had not switched from cigarettes.

Discussion

Interpretation of results

Our results indicate that current pipe or cigar smokers who switched from cigarettes smoke about the same amount of tobacco as pipe and cigar smokers who never smoked cigarettes but that they tend to inhale more. Most of the reduction in the risk of dying from ischaemic heart disease, lung cancer, and chronic obstructive lung disease combined compared with that in continuing cigarette smokers is attributable to the fact that pipe and cigar smokers smoke less tobacco than the cigarette smokers (median 8.1 g a day compared with 20 g a day). The risk of dying from the three diseases combined in these switchers was less (24% less; 95% confidence interval 55% less to 27% more) than in light cigarette smokers who smoked the same amount of tobacco—that is, those who smoked between one and 14 cigarettes a day with a median of 8 g tobacco a day—so the reduction may also be explained in part by reduced inhaling. There may be other differences in lifestyle between the switchers and non-switchers, though this is probably unlikely because adjustment for serum cholesterol concentration and systolic blood pressure made no difference to the risk estimates.

Our results indicate a specific adverse effect among pipe smokers who switched from cigarettes that is not due to former cigarette smoking. The carboxyhaemoglobin saturations shown in table 1 suggest this is due to increased inhaling. For example, the mean carboxyhaemoglobin saturation in pipe smokers who never smoked cigarettes was about twice the background level (1.4 *v* 0.7); it was nearly three times the background level in those who switched from cigarettes (1.9 *v* 0.7). An interesting result, though one that is not directly relevant to the question we sought to answer, was that carboxyhaemoglobin saturation was related to the risk of the three specified smoking related diseases independently of smoking category or amount smoked. Indeed, after adjustment for carboxyhaemoglobin saturations, smoking was no longer significantly related to risk. We estimated that regardless of smoking category, the risk of dying of the three specified diseases increased by 22% per 1% increase in carboxyhaemoglobin saturation.

Previous studies

Two previous studies have compared mortality in pipe and cigar smokers who switched from smoking cigarettes with those who never smoked cigarettes, but neither yielded conclusive results. The paper by Kaufman et al was a case-control study of cigar and pipe smoking in relation to myocardial infarction in

young men (aged 40-54 years).[10] The group of men who stopped smoking cigarettes and switched to pipes or cigars included men who could have switched as recently as two years previously, so that any excess risk could have been associated with the residual effect of cigarette smoking rather than the effect of pipe or cigar smoking. The Whitehall study showed that pipe or cigar smokers who previously smoked cigarettes had a higher mortality than pipe or cigar smokers who did not previously smoke cigarettes,[11] though again this result could have been due to the residual effects of cigarette smoking in the former cigarette smokers. The study did show that cigarette smokers who switched to smoking pipes (but not to smoking cigars) had a higher mortality from all causes than former cigarette smokers (relative risk 1.17; 1.03 to 1.34), with a significant increase in risk of ischaemic heart disease. The difference was explained by the pipe smokers' previous cigarette smoking habits.

Public health implications

Our results have public health implications. Cigarette smokers may be able to stop smoking when they receive advice and support and by using aids such as nicotine chewing gum.[12] Some, however, may still have difficulty in giving up smoking altogether, and these smokers would be better off changing to cigars or pipes instead of continuing to smoke cigarettes. Much of the effect is due to the reduction in the quantity of tobacco smoked; the rest may be attributable to reduced inhalation. Cigarette smokers may find it easier to reduce consumption by changing to smoking cigars or pipes rather than by smoking fewer cigarettes. The risk of the three specified smoking related diseases, none the less, is still higher than in men who only ever smoked pipes or cigars, higher than in those who gave up smoking altogether—and, of course, higher than in men who never smoked at all.

We thank Sir Richard Doll, Leo Kinlen, Jim Haddow, and Malcolm Law for their helpful comments.

Funding: BUPA Medical Research and Development Ltd gave financial support to the BUPA Epidemiological Research Group within the Wolfson Institute of Preventive Medicine.

Conflict of interest: None.

Key messages

- The health risks from smoking pipes or cigars are less than those from smoking cigarettes, but there is little direct evidence on these risks in cigarette smokers who switch to pipes or cigars
- This prospective study shows that smokers who switch from cigarettes to pipes or cigars halve their combined risk of dying of lung cancer, ischaemic heart disease, or chronic obstructive lung disease compared with continuing smokers, but their risk was still about 50% higher than that of lifelong non-smokers
- Some of this reduction in risk was due to reduced inhaling, but most of it was due to a reduction in the amount of tobacco smoked
- The best option is either not to smoke or to give up altogether; failing that, switching to pipes or cigars is better than continuing to smoke cigarettes

1 Rodenstein DO, Stenescu DC. Pattern of inhalation of tobacco smoke in pipe, cigarette, and never smokers. *Am Rev Respir Dis* 1985;132:628-32.
2 Goldman AL. Carboxyhaemoglobin levels in primary and secondary cigar and pipe smokers. *Chest* 1977;72:33-5.
3 Ockene JK, Pechacek TF, Vogt T, Svendsen K. Does switching from cigarettes to pipes or cigars reduce tobacco smoke exposure? *Am J Public Health* 1987;77:1412-6.
4 Castleden CM, Cole PV. Inhalation of tobacco smoke by pipe and cigar smokers. *Lancet* 1973;i:21-2.
5 Turner JAM, Sillett RW, McNicol MW. Effect of cigar smoking on carboxyhaemoglobin and plasma nicotine concentrations in primary pipe and cigar smokers and ex-cigarette smokers. *BMJ* 1977;ii:1387-9.
6 Pechacek TF, Folsom AR, de Gaudermaris R, Jacobs DR, Luepker RV, Gillum RF, et al. Smoke exposure in pipe and cigar smokers. Serum thiocyanate measures. *JAMA* 1985;254:3330-2.
7 Cowie J, Sillett RW, Ball KP. Carbon monoxide absorption by cigarette smokers who change to smoking cigars. *Lancet* 1973;i:1033-5.
8 Wald NJ, Law M, Watt H, Wu T, Bailey A, Johnson M, et al. Apolipoproteins and ischaemic heart disease: implications for screening. *Lancet* 1994;343:75-9.
9 Wald N, Idle M, Bailey A. Carboxyhaemoglobin levels and inhaling habits in cigarette smokers. *Thorax* 1978;33:201-6.
10 Kaufman DW, Palmer JR, Rosenberg L, Shapiro S. Cigar and pipe smoking and myocardial infarction in young men. *BMJ* 1987;294:1315-6.
11 Ben-Shlomo Y, Davey Smith G, Shipley MJ, Marmot MG. What determines mortality risk in male former cigarette smokers? *Am J Public Health* 1994;84:1235-42.
12 Tang JL, Law M, Wald N. How effective is nicotine replacement therapy in helping people to stop smoking? *BMJ* 1994;308:21-6.

(Accepted 10 April 1997)

A memorable patient

When all else fails examine the patient

It was an extremely busy receiving night on the surgical service, which in retrospect is my only inadequate excuse for what occurred. As the senior registrar, I was coping with most of the 15 or 16 emergencies admitted through the night, including patients with perforated ulcers, acute appendicitis, ischiorectal abscess, blunt abdominal trauma, upper gastrointestinal haemorrhage, acute pancreatitis, and acute urinary retention. In between cases in the operating theatre I examined an elderly lady on the ward at about 3 00 am who had been admitted with a short history of cramping abdominal pain. The abdomen was slightly distended, with no signs of peritoneal irritation, rectal examination was normal, and the findings on abdominal radiography were consistent with early small bowel obstruction. After my hurried examination I ordered drip and suction while I rushed back to the long list of emergency operations already scheduled.

Eventually, the operating list was completed and there was just time for a bath and some breakfast before rounds began with the chief and the entire entourage of students, house staff, registrars, and consultants. As we circled the old Florence Nightingale ward, I described with some self satisfaction the salient features of each case and how it had been handled, while the chief paused here and there to examine an abdomen, check an incision, make a management suggestion, or teach on a point of special interest. When we came to the bedside of the lady described above, I explained the history and findings, and how I had decided to treat her with intravenous fluids and nasogastric suction pending a more definitive diagnosis. With a sonorous "hrrumph," the chief threw the bed covers down in order to examine the abdomen. To my horror, I and everyone else in the assembled multitude immediately saw a lump of approximately 3 cm diameter low in the right groin. After a pregnant pause, every moment adding to my extreme discomforture, he said "I suppose Dr Wright will now give us an erudite dissertation on the non-operative treatment of strangulated femoral hernia." I never needed to be reminded thereafter that adequate abdominal examination includes "nipples to knees."

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Risk Perception and Cigar Smoking Behavior

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***Objective*: To examine the cigar smoking perceptions and behaviors of US adults. *Methods*: A national sample of 1,012 adults was interviewed by telephone. *Results*: Current cigar smokers differed from nonsmokers in perceptions of personal risk for cancer and views about cigar smoking as a cancer cause. Both groups showed substantial acceptance of the glamorized image of cigar smokers. *Conclusion*: Although recognizing smoking as a cancer cause in general, cigar smokers tended to show a self-exempting "optimistic bias" with regard to perceptions of their own risks.**

***Am J Health Behav* 2001;25(2):106-114**

There is growing evidence that cigar smoking causes cancer of the lung, oral cavity, larynx, esophagus, and possibly cancer of the pancreas.[1-4] Nevertheless, stimulated by ample and effective advertising, in the last several years, cigar smoking has increased dramatically.[5] The tobacco industry proudly reports an increase of 50% or more in the sale of premium cigars in the United States.[6]

The US Department of Agriculture estimates that cigar sales in the United States increased by almost 50% between 1993 and 1997.[7] An article in the *Morbidity and Mortality Weekly Review* (*MMWR*) suggests that the prevalence of cigar smoking among youth in the United States is as high as 27%.[8] However, the current prevalence rates for adults with regard to cigar smoking are unclear because of a lack of recent studies that focus on assessing this type of tobacco use in the general adult population.

Glossy magazines have appeared that regularly feature celebrities, including movie stars and sports figures, to promote cigar smoking as a glamorous activity enjoyed by successful and accomplished men and women. The increase in cigar smoking has been attributed to the success of this campaign and the resulting improvement in the public's perception of cigar smoking.[6]

It appears that many people consider cigars to be safe because they do not inhale cigar smoke, and they think that compared to cigarettes, cigars have almost no risk. For example, considerable discussion concerning the relative safety of cigars is found on the Internet, and this view of the relative safety of cigars is commonly exhibited. Models that attempt to explain health behaviors tend to emphasize the role of perceptions of health risks over the actual risks as supported by epidemiological data.[9,10] Studies have shown that people who continue smoking tend to consider their own risks from

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smoking to be lower than the risks of other smokers.[11,12] This pattern of estimating the risk from a health hazard for oneself as being lower than the risk for the broader category to which one belongs has been called "optimistic bias."[10] Weinstein[13] has observed that the magnitude of this unrealistic optimism varies from hazard to hazard but is particularly large for problems that are believed to be preventable, such as lung cancer. A number of research studies have shown that cigarette smokers who believe their risks from smoking are lower than the risks for other smokers use a variety of rationalizations to explain their reduced risk.

The association of tobacco smoking with attitudes and risk perceptions has been described in relation to cigarettes, but few data are available on the public's perceptions of risks of cigar smoking.[14] Research data are also lacking concerning the general public's actual perceptions of cigar smokers.

The purposes of this article are to describe the results of a national survey with regard to the association of health-risk perceptions to cigar smoking and to describe the current perceptions that the public has regarding cigar smokers.

METHOD

Subjects and Procedure

The data reported here were collected through a telephone survey conducted between July 31 and August 3, 1997, among a national sample composed of 1,012 adults (504 men and 508 women) who were 18 years of age or older and living in private households in the continental United States. The survey was completed over a 4-day period from Thursday through Sunday. Calls on Thursday and Friday were made in the evenings up to 9:00 pm local time and on Saturday and Sunday all day up to 9:00 pm, or until all quotas were filled. The data were gathered by the Opinion Research Corporation International employing an unrestricted random-sampling procedure that was used to generate a random-digit-dialing sample that controlled the amount of serial bias found in systematic sampling. For those phone numbers selected for the sample, up to 4 attempts were made to obtain a completed interview. The sample was fully replicated and stratified by region, and only one interview was conducted at each household contacted.

The interviews were conducted by trained interviewers who employed a computer-assisted telephone interviewing (CATI) system.

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Measures

Cigar and cigarette smoking behavior were assessed using a modification of the question format used by the Office of Population Censuses and Surveys to study smoking in young adults in 21 European countries.[14] Respondents were asked to describe their cigar smoking habits using one of 8 categories:

1. I have never smoked a cigar, not even a puff.
2. I have only ever tried 1 or 2 cigars.
3. I used to smoke cigars sometimes, but not now.
4. I used to smoke cigars regularly, but not now.
5. I smoke cigars, but not as many as one a month.
6. I usually smoke between 1 and 10 cigars per month.
7. I usually smoke between 10 and 20 cigars per month.
8. I usually smoke more than 20 cigars per month.

A similar question was used to ask about the respondent's cigarette smoking habits, except that instead of "per month," the time period was changed to "per day" (for categories 5, 6, 7, and 8). For both the question about cigar smoking and the one about cigarette smoking, responses in categories 1, 2, 3, and 4 were categorized as current nonsmokers, whereas responses in categories 5, 6, 7, and 8 were categorized as current smokers.

Respondents who placed themselves in categories 3 to 8, indicating that they had smoked or were still smoking cigars, were

A total of 93 respondents currently smoked cigars, and 271 smoked cigarettes.

also asked to indicate how many years they had smoked cigars. The same format was used to ask about years of cigarette smoking. Those who indicated they had smoked cigars and/or cigarettes were asked how old they were when they began smoking.

Five risk-perception questions used a scale of 0 (*no chance*) to 10 (*absolutely certain*) to ask respondents to rate their own chances in the next 20 years of "developing cancer." (Other risk-perception questions included their chance of contracting HIV, having an automobile accident, suffering a heart attack, and suffering a stroke.) Three risk questions dealing with the risk of developing specific types of cancer asked respondents to use a number from 0 to 100 to indicate how many out of 100 cigar smokers they thought would develop: (a) "lung cancer," (b) "lip and/or mouth cancer," and (c) "throat cancer."

Nine questions dealt with the perceptions of those interviewed regarding 3 different "health factors and health behaviors that may or may not cause cancer." Respondents rated (a) "environmental conditions," (b) "genetic factors," and (c) "stress" on a scale from 0 to 10. They were told: "On this scale, a health factor that DEFINITELY causes cancer would be rated 10 and a health factor that DEFINITELY DOES NOT cause cancer would be rated 0." In order to minimize order effects, interviewers rotated the sequence in which these factors and the conditions listed in each of the questions above were read. Respondents were also asked how they would rate various health behaviors as causes of cancer. These behaviors included smoking cigarettes, smoking cigars, inhaling secondhand cigarette smoke, and inhaling secondhand cigar smoke. As with the health factors, a scale of 0 to 10 was used for these ratings, and the sequences were rotated to minimize order effects. (Other questions asked the respondent to rate using smokeless tobacco and drinking alcoholic beverages as causes of cancer.)

Seven questions dealt with the perceptions that people have of typical cigar smokers. The respondent was told: "The next few questions will ask you to describe the characteristics of the typical CIGAR smoker. Out of 100 cigar smokers, how many

1. are athletic,
2. are female,
3. are managers or executives,
4. are over the age of 40,
5. are under the age of 18,
6. have graduated from college,
7. make more than $50,000 a year.

Data Analysis

A total of 93 respondents currently smoked cigars, and 271 smoked cigarettes. There were 692 in the group who smoked neither cigars nor cigarettes (current nonsmokers) and a total of 51 who currently smoked both. In this study, comparisons are made between those who are current cigar smokers (n=93), regardless of their current cigarette smoking habits, and those who are current nonsmokers (n=692). Thus, results are reported for only 785 of the 1,012 respondents.

Exploratory data analysis showed that the responses to risk questions on both the 0 to 10 and the 0 to 100 scales were not normally distributed ($p<.0001$ for all variables). Data for most questions using the 0 to 10 scale were severely skewed to the right, whereas data on the 0 to 100 scale in general were skewed to the left and were not unimodal. For analysis of data with pronounced nonnormality, parametric statistics such as means, t-tests, and ANOVA are not recommended.[15-17] Therefore, it was decided to analyze the data using nonparametric methods such as medians, proportions, and chi-square tests. Data on the 0 to 100 scale were treated as continuous, with medians and median-split chi-square tests being used to compare current cigar smokers with current nonsmokers. Data on the 0 to 10 scale were treated as categorical, given their much more limited range of values. Three categories were created to obtain proportions and chi-square comparisons. Scores of 0, 1, or 2 formed a "low" perceived risk or causation category; scores of 3 to 7 formed an "intermediate" per-

TABLE 1
Characteristics of Current Cigar Smokers and Current Nonsmokers

Characteristic		Current Nonsmokers N	Current Nonsmokers %	Current Cigar Smokers N	Current Cigar Smokers %
Age**	18-24	60	8.8	26	28.3
	25-44	278	40.5	47	51.1
	45-54	132	19.3	12	13.0
	55-64	93	13.6	6	6.5
	65 or more	122	17.8	1	1.1
Male**		326	47.1	83	89.2
Race/Ethnicity	White	556	81.8	77	84.6
	Black	73	10.7	10	11.0
	Asian	12	1.8	2	2.2
	Other	39	5.7	2	2.2
Hispanic		52	7.6	6	6.6
Education	Less than high school graduate	62	9.2	10	11.2
	High school graduate	209	31.1	27	30.3
	Some college	162	24.1	24	27.0
	College graduate or higher	239	35.6	28	31.5
Income	Less than $10,000	32	5.4	4	5.0
	$10,000 – 24,999	103	17.5	15	18.8
	$25,000 – 49,999	241	40.9	29	36.2
	$50,000 or more	213	36.2	32	40.0
Occupation	Professional/managerial/owner	219	47.2	42	56.7
	Sales, clerical	80	17.3	7	9.5
	Craftsman/foreman	74	16.0	13	17.5
	Semiskilled/unskilled	47	10.2	5	6.8
	Service worker	42	9.1	6	8.1
	Other	1	0.2	1	1.4
Marital Status**	Married/marriage-like relationship	428	62.1	44	47.9
	Single, never married	132	19.2	35	38.0
	Divorced/separated	76	11.0	12	13.0
	Widowed	53	7.7	1	1.1

**** p<.01 for chi-square test of difference between groups.**

ceived risk or causation category; and scores of 8, 9, or 10 formed a "high" perceived risk or causation category. SUDAAN was used for data analysis because it estimates standard errors for all point estimates. The sampling design, however, was described as simple random sampling.[18] To correct for the probability of spurious results because of multiple statistical tests, the overall alpha to be considered as statistically significant in discussing the results was set at the level of p<.01 rather than p<.05.

RESULTS

Current Cigar and Cigarette Smoking Prevalence

The self-reports of smoking behavior

TABLE 2
Perceptions of Personal Risk for Cancer and Cancer Causation by Current Smoking Status, US Adults

Question	Current Smoking Status (n)	% (se) who perceive risk / causal effect as† Low	Intermediate	High
Your own risk for cancer in the next 20 years	Nonsmoker (666)	29.0 (1.8)	59.1 (1.9)	11.9 (1.3)
	Cigar smoker (91)	19.7 (4.3)	71.6 (4.8)	8.7 (2.8)
Do environmental conditions cause cancer?	Nonsmoker (682)	6.9 (1.0)	46.2 (1.9)	46.9 (2.0)
	Cigar smoker (93)	12.8 (3.4)	48.5 (5.3)	38.7 (5.2)
Do genetic factors cause cancer?	Nonsmoker (679)	7.1 (1.0)	42.8 (1.9)	50.1 (2.0)
	Cigar smoker (93)	11.6 (3.4)	37.7 (5.1)	50.7 (5.3)
Does stress cause cancer?	Nonsmoker (680)	31.6 (1.8)	48.5 (2.0)	19.9 (1.6)
	Cigar smoker (93)	38.9 (5.1)	49.4 (5.3)	11.7 (3.4)
Does smoking cigars cause cancer?****	Nonsmoker (684)	2.9 (0.7)	18.0 (1.5)	79.1 (1.6)
	Cigar smoker (92)	6.8 (2.6)	46.6 (5.3)	46.6 (5.3)
Does secondhand cigar smoke cause cancer?****	Nonsmoker (683)	6.1 (1.0)	29.6 (1.8)	64.3 (1.9)
	Cigar smoker (92)	18.8 (4.0)	47.1 (5.3)	34.1 (5.1)

† **Risk/causal effect responses of 0 to 10 was categorized as follows: Low = response of 0,1 or 2; Intermediate = response of 3,4,5,6 or 7 and; High = response of 8,9 or 10.**
****** P-value for Chi-square testing difference between current cigar smokers and current nonsmokers <.0001.**

from the telephone interview sample indicated an overall sample prevalence of 9.4% (95% CI ±1.8) as current cigar smokers, with more than 8 times more men smoking cigars (17.3%, 95% CI ±3.4) than women did (2.0%, 95% CI ±1.3). The sample prevalence of cigarette smoking of 27.4% (95% CI ±2.8) was similar to that reported in other studies.[19]

Sample Characteristics

Table 1 presents demographic data on the sample of current cigar smokers and nonsmokers who are included in this data analysis (n=785). Overall, the majority of interviewees were in the 25 to 44 and 45 to 54 age categories. The total sample was 82.1% white, 10.8% black, and 7.1% Asian American and other minorities; 7.5% were Hispanic. With regard to education, overall, about 10% were less than high school graduates, about a third gave high school graduation as their highest level of education, and somewhat more than half had completed at least some college. About two-thirds of the total sample were employed, with almost half working in professional occupations. About a quarter of the sample earned less than $25,000, whereas the median household income was between $35,000 and $39,999. As shown on Table 1, the 2 groups (current cigar smokers and current nonsmokers) did differ statistically on age, gender, and marital status, but not race/ethnicity, educational attainment, income, or occupation. Current cigar smokers were younger than current nonsmokers and were more likely to be male and unmarried.

Perceptions of Personal Risk for Cancer, Cancer Causation, and Characteristics of Cigar Smokers

Table 2 presents a series of chi-square analyses of perceptions of personal risk for cancer and cancer causation comparing those who indicated they were current cigar smokers with those who said they were not currently smoking. Those who currently smoked cigars tended to see their risk of developing cancer in the

TABLE 3
Perceived Risk for Smokers Developing Specific Cancers by Current Smoking Status, US Adults

Question	Current Smoking Status (n)	Median (se)
How many of 100 cigar smokers will develop lung cancer? ***	Nonsmoker (670) Cigar smoker (91)	45.9 (0.8) 21.2 (5.1)
How many of 100 cigar smokers will develop lip/mouth cancer?	Nonsmoker (665) Cigar smoker (91)	29.8 (2.4) 29.2 (3.9)
How many of 100 cigar smokers will develop throat cancer?	Nonsmoker (666) Cigar smoker (91)	29.9 (3.0) 26.2 (5.1)

***** P-value of χ^2 median split test for difference in medians between current cigar smokers and current nonsmokers <.001**

next 20 years as being at an intermediate level (defined as 3, 4, 5, 6, or 7 on the 10-point scale). Those not currently smoking tended to see their risk as lower, although there was also a greater proportion of nonsmokers (11.9%) versus current cigars smokers (8.7%) who saw themselves as being at higher risk (as 8, 9, or 10 on the scale). These differences, however, were not statistically significant (p= .062).

With regard to factors that cause cancer, the chi-squares comparing current cigar smokers with nonsmokers were not statistically significant with regard to perceptions of environmental conditions, genetic factors, or stress. However, ratings of various health behaviors as causes of cancer showed significant differences between cigar smokers and nonsmokers in perception of causality. A greater proportion of nonsmokers (79.1%) than current cigar smokers (46.6%) gave a high rating to the role of cigar smoking in causing cancer; similarly, nearly half of cigar smokers (46.7%) viewed the contribution as only intermediate, compared to about a fifth of nonsmokers (18.0%). Perceptions of the role of secondhand cigar smoke in the etiology of cancer also differed at a statistically significant level, with three fifths (64.3%) of those not currently smoking giving this a high rating compared to only a little more than a third (34.1%) of cigar smokers.

Because the 2 groups did differ on age, gender, and marital status, additional chi-square analyses were performed to investigate the influence of these variables on the associations. In univariate testing, only gender, not age or marital status, was found to have a statistically significant association with level of perceived cancer risk of smoking cigars and secondhand cigar smoke. Chi-square analyses for these 2 risks stratified on gender still found a statistically significant association with smoking group (p=.001 for both analyses), even after difference in gender of the 2 groups was taken into account.

In Table 3, median-split chi-square comparisons regarding perceptions of how many out of a 100 cigar smokers would develop cancer at 3 different sites are presented for cigar smokers compared to nonsmokers. There was a statistically significant difference between cigar smokers and nonsmokers with regard to lung cancer, but not for lip/mouth cancer or throat cancer. Again, additional chi-square analyses were performed to determine the role of differences in age, gender, and marital status on this association. In univariate testing, only gender was found to have a statistically significant association with median number of cigar smokers who would develop lung cancer. Analysis stratified on gender, however, found that the association of smoking status and this variable was no longer statistically significant (p=.07) once differences in gender were accounted for in the analysis.

As shown in Table 4, cigar smokers perceived a somewhat larger number of

TABLE 4
Perceived Personal Characteristics of Cigar Smokers by Current Smoking Status, US Adults

How many of 100 cigar smokers are:	Current Smoking Status (n)	Median (se)
Athletic*	Nonsmoker (651)	18.1 (1.4)
	Cigar smoker (93)	23.4 (1.9)
Female	Nonsmoker (659)	9.0 (0.2)
	Cigar smoker (92)	8.6 (1.2)
Managers or Executives	Nonsmoker (653)	46.5 (0.5)
	Cigar smoker (93)	39.2 (4.4)
Over age 40	Nonsmoker (653)	49.5 (0.2)
	Cigar smoker (93)	48.0 (1.0)
Under Age 18	Nonsmoker (646)	6.7 (0.9)
	Cigar smoker (92)	7.2 (1.0)
College Graduates	Nonsmoker (649)	47.4 (0.4)
	Cigar smoker (90)	49.2 (0.6)
Earning >$50,000/yr	Nonsmoker (642)	47.0 (0.4)
	Cigar smoker (91)	49.2 (2.6)

*** P-value of χ^2 median split test for difference in medians between current cigar smokers and current nonsmokers <.05**

"typical cigar smokers" to be athletic (23 of 100) than did nonsmokers (18 of 100), although this difference was not statistically significant at the α= .01 level. There were also no statistically significant differences in the median number of cigar smokers who were perceived to be female, a manager or an executive, over 40, under 18, college graduates, or earning over $50,000 annually. Both groups indicated that only about 9 out of 100 cigar smokers would be female. They judged that about two fifths would be executives; about half would be over 40 and less than one tenth under 18; and about half would be college graduates or earning $50,000 or more per year.

DISCUSSION AND CONCLUSIONS

These data show that current cigar smokers and those not smoking differ in their perceptions of personal risk for cancer and in their views about the role of cigar smoking in causing cancer. Cigar smokers do acknowledge that they are at some level of risk for developing cancer in the next 20 years, because almost three quarters see themselves as being at an intermediate level of risk. They also recognize that smoking cigars causes cancer, because almost half view cigar smoking as a high-level cause of cancer. However, they do not apply these same causal perceptions to themselves, because only about 9% rate their personal risk for cancer as high. Thus, they seem to show the same "optimistic bias" in comparing their risk to others' risk that has been described by previous researchers in studies of community samples' perceptions of their susceptibility to smoking-related cancer and other health problems.[11,12] By exempting themselves from the risks of cancer, cigar smokers may reduce cognitive dissonance produced by the inconsistency between their beliefs about the health risks of smoking and their own smoking behavior.

Cigar smokers also recognize the role of secondhand smoke as a cause of cancer, with over a third attributing relatively high risk to environmental cigar

smoke. However, this proportion is considerably less than the nearly 65% of nonsmokers who rate secondhand cigar smoke as having a high causal effect. Again, there is a significantly smaller breadth of appreciation for cancer risk from secondary smoke among those who are currently smoking cigars.

Cigar smokers and nonsmokers do not differ in their perceptions of the contribution that environmental, genetic, and stress factors make to the development of cancer, but they do differ in their views regarding what proportion of cigar smokers will develop lung cancer. On average, cigar smokers believe that about a fifth of cigar smokers are likely to develop lung cancer, whereas nonsmokers believe that significantly more (45 out of 100) cigar smokers will develop cancer in this site. This association with smoking status was not statistically significant, however, once gender was taken into account. Both cigar smokers and nonsmokers see smoking cigars as contributing to the development of lip/mouth cancer or throat cancer in only about a third of cigar smokers.

Both cigar smokers and nonsmokers appeared to accept the more glamorized view of cigar smokers in that they viewed about two fifths to a half of typical cigar smokers as relatively well-to-do, educated, older, managers or executives. Neither group viewed cigar smokers as including very many women or teenagers, whereas both groups viewed about a fifth of cigar smokers as athletic.

The results of this study indicate that cigar smokers as compared to nonsmokers are inclined to minimize the role of smoking cigars in cancer causation. The results also show that cigar smokers, although recognizing that cigar smoking in general does make some contribution to increased cancer risk, also tend to minimize their own estimated risks for developing cancer.

Recent reviews have noted the limitations of communicating risk information in probabilistic terms.[20-22] Lay conceptions of a given health risk are typically based on more than the numerical probability of a given event; they involve a more complex array of cognitive and affective beliefs.[20] Providing more contextualized information about the causes of a health problem, the severity of the consequences, and what can be done to prevent or treat the problem enables people to develop a model that places the risk in a personal context of individual relevance. Rothman and Kiviniemi have recommended the use of contextually based interventions dealing with the causes and consequences of a health problem in combination with precise information on probability of an individual's developing that problem. However, given the limitation in people of their ability to interpret numerical information about a specific health risk, people would also have to be taught basic lessons in how to understand probability. However, this type of intervention is yet to be tested systematically and poses a challenge for future research on risk communication.

Smoking cigars is perhaps one of the most accessible and affordable status symbols or behaviors available for young Americans.

This survey found that the highest prevalence of current cigar smoking was among younger males (33.7% in the 18 to 24 years category reported current cigar smoking, compared to 17.8% in the 25 to 44 age group, 12.6% each in the 45 to 54 and 55 to 64 age-groups, and 1.6% among those 65 and over). Smoking cigars is perhaps one of the most accessible and affordable status symbols or behaviors available for young Americans. This might explain why the current cultural image of a cigar smoker is one of a more successful middle-aged male, although cigar smoking is actually most prevalent in younger males.

Only 93 of the total sample reported that they were current cigar smokers. Of these, 51 also currently smoked cigarettes. Because some of the cigar smokers smoked cigarettes, the issue of whether these individuals differed in their perceptions from those who only smoked cigars is relevant for consideration. However, to do analyses comparing those who smoke only cigars with those who smoke both cigarettes and cigars would require a larger sample of both groups to have sufficient statistical power.

In summary, this study has shown that current cigar smokers differ from nonsmokers in their perceptions of personal risk and in their views about the role of smoking cigars in the etiology of cancer. Although cigar smokers recognize the role of smoking as a cause of cancer to some degree, they do not apply these same risk perceptions to themselves. Finally, we have found that both those who actually smoke cigars and nonsmokers see cigar smokers in terms of current cultural stereotypes projected by the media. Clearly, perceptions of cigar smokers tend to reflect media images as well as a tendency toward a self-serving optimistic bias that must be taken into account in developing prevention programs to convince cigar smokers to give up smoking to reduce their health risks. ■

REFERENCES

1. Shanks TG, Burns DM. Disease consequences of cigar smoking. In: National Institutes of Health/National Cancer Institute, ed. Cigars: Health Effects and Trends. Bethesda, MD; US Dept of Health, Education, and Welfare, Public Health Service, 1998.
2. Baker F, Ainsworth SR, Dye JT, Crammer C, Thun MJ, et al. Health risks associated with cigar smoking. *JAMA* 2000;284:735-741.
3. Boffetta P, Pershagen G, Joeckel K-H, Forastiere F, Gaborieau V, et al. Cigar and pipe smoking and lung cancer risk: a multi-center study from Europe. *J Natl Cancer Inst* 1999;91(8):697-701.
4. Shapiro JA, Jacobs EJ, Thun MJ. Cigar smoking in men and risk of death from tobacco-related cancers. *J Natl Cancer Inst* 2000;92(4):333-337.
5. Slade J. Marketing and promotion of cigars. In: National Institutes of Health/National Cancer Institute, ed. Cigars: Health Effects and Trends. Bethesda, MD; US Dept of Health, Education, and Welfare, Public Health Service, 1998: 195-219.
6. O'Hara CB. Cohabitation: how the cigar resurgence is affecting the cigarette industry. *Tobacco International* 1996;198:38-41.
7. US Dept of Agriculture. Tobacco Situation and Outlook Report. Washington, DC; US Department of Agriculture, Commodity Economics Division, Economic Research Service,1997. TBS-239.
8. Eriksen MP, Kaufman N, Cummings KM, Connally G. Cigar smoking among teenagers — United States, Massachusetts, and New York. *MMWR* 1997;46:433-440.
9. Becker MH. The health belief model and personal health behavior. *Health Education Monograph* 1974;2:324-473.
10. Weinstein ND. Unrealistic optimism about susceptibility to health problems: conclusions from a community-wide sample. *J Behav Med* 1987;10(5):481-500.
11. McCoy SB, Gibbons FX, Reis TJ, Gerrard M, Luus CAE, et al. Perceptions of smoking risk as a function of smoking status. *J Behav Med* 1992;15:469-488.
12. Strecher VJ, Kreuter MW, Kobrin SC. Do cigarette smokers have unrealistic perceptions of their heart attack, cancer, and stroke risks? *J Behav Med* 1995;18(1):45-54.
13. Weinstein ND. What does it mean to understand a risk? evaluating risk comprehension. *Journal of the National Cancer Institute Monographs* 1999;25:15-20.
14. Steptoe A, Wardle J, Smith H, Kopp M, Skrabski A, et al. Tobacco smoking in young adults from 21 European countries: association with attitudes and risk awareness. *Addiction* 1995;90:571-582.
15. Blalock HM. Social Statistics. New York: McGraw Hill; 1979.
16. Keppel G. Design and Analysis: A Researcher's Handbook. Upper Saddle River, NJ: Prentice Hall; 1991.
17. Neter J, Kutner MH, Nachtsheim CJ, Wasserman W. Applied Linear Statistical Models. Chicago: Irwin; 1996.
18. Shah BV, Barnwell BG, Bieler GS. SUDAAN User's Manual, Release 7.5. Research Triangle Park, NC: Research Triangle Institute; 1997.
19. Giovino GA, Schooley MW, Zhu BP, Chrismon JH, Tomar SL, et al. Surveillance for selected tobacco-use behaviors - United States 1900-1994. *Morbidity and Mortality Weekly Review* 1994;43:1-43.
20. Rothman AJ, Kiviniemi MT. Treating people with information: an analysis and review of approaches to communicating health risk information. *Journal of the National Cancer Institute Monographs* 1999;25:44-51.
21. Fischhoff BP. Why (cancer) risk communication can be hard. *Journal of the National Cancer Institute Monographs* 1999;25:7-13.
22. Leventhal H, Kelly K, Leventhal EA. Population risk, actual risk, perceived risk, and cancer control: a discussion. *Journal of the National Cancer Institute Monographs* 1999;25:81-84.

Nicotine & Tobacco Research, 2017, 1351–1358
doi:10.1093/ntr/ntw244
Original investigation
Received December 21, 2015; Editorial Decision September 12, 2016; Accepted September 21, 2016

Original investigation

Risk Perceptions of Little Cigar and Cigarillo Smoking Among Adult Current Cigarette Smokers

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Abstract

Introduction: Few studies have examined the perceptions of risk of little cigar and cigarillo (LCC) smoking among cigarette smokers, which is important for expanding regulatory policies and developing prevention programs. We examined current cigarette smokers' perceived harm of LCC smoking, and determined whether these perceptions were associated with susceptibility and intention to continue smoking LCCs.

Methods: Data were from the 2014 Tobacco Products and Risk Perceptions Survey of a probability sample of 5717 US adults. Data were analyzed for a subsample of 1191 current cigarette smokers who were stratified into three groups: (1) dual current cigarette smokers who had ever used LCCs, (2) current smokers susceptible to LCC smoking, and (3) current smokers who were not susceptible to LCC smoking.

Results: Overall, 47.2% of participants were dual smokers, 12.7% were susceptible to LCC smoking, and 40.1% were not susceptible. Perceptions of risk of LCCs varied across the groups. Dual smokers were more likely to perceive that daily LCC smoking is "very risky" (*OR* = 1.64, 95% CI = 1.08, 2.41) while occasional LCC smoking is only "somewhat risky" (*OR* = 1.71, 95% CI = 1.02, 2.87). Of the dual smokers, 20.7% intended to continue smoking LCCs in the future. Perceptions of addiction and risk of daily LCC smoking significantly predicted intention to continue LCC smoking. Addiction perceptions also significantly predicted susceptibility to initiate LCC smoking.

Conclusions: Perceptions about harms from and addiction to LCCs could predict future LCC smoking. Health communication campaigns need to address the harms of LCCs.

Implications: Our data suggest that perceptions of risk about the addictiveness of LCCs and frequency of use are important determinants of the LCC smoking susceptibility among some cigarette smokers and intended continued use among cigarette smokers with a history of LCC use. Health communication campaigns should address misperceptions related to LCCs.

Introduction

As cigarette smoking continues to decline, use of other combustible tobacco products among adults is increasing.[1] Little cigars and cigarillos (LCCs) are cigarette-like products that are often flavored,[2] heavily marketed, and are inexpensive alternatives to cigarettes.[3] LCCs are often used concurrently with cigarettes or other tobacco products.[4–6] According to the 2012–2013 National Adult Tobacco

Survey (NATS), 18.4% of adult cigar users reported smoking little filtered cigars as their usual type smoked, while 61.8% reported smoking cigarillos. Nearly 60% of these adult cigar smokers were also current or former cigarette smokers.[7] Though reasons for LCC use are not well documented, some adult smokers may dual use LCCs to either quit smoking or to complement or substitute for cigarette use.[8] Regardless, dual use of cigarettes and LCCs may prolong smoking, perpetuate nicotine addiction, and lead to detrimental health effects on users, particularly if use is sustained over a long period of time.

The Family Smoking Prevention and Tobacco Control Act (FSPTCA) of 2009 provided the Food and Drug Administration (FDA) with the authority to regulate the manufacturing, distribution, and marketing of cigarettes. In May 2016, the FDA announced that it would extend its regulatory authority to include LCCs and other tobacco products. Though bringing LCCs under the regulatory authority of the FDA is a critical step to protect the public's health, it is important to note that LCCs are not as strictly regulated as cigarettes. Unlike cigarettes, LCCs are available in characterizing flavors, such as vanilla and blueberry. Flavor additives in tobacco products are known to appeal to smokers, and a substantial proportion of adult smokers report flavored cigar use. King and colleagues found that among adult cigar smokers aged 18 and older who completed the 2009–2010 NATS, 42.9% reported flavored cigar use. The prevalence of flavored cigar use was greater among females, those younger in age, Hispanics, and Other, non-Hispanics.[9] The less-regulated status of LCCs may unintentionally fuel inaccurate perceptions about the relative harm of LCCs compared to cigarettes, especially among vulnerable populations such as young adults and some racial/ethnic minority groups.

Cigarette smokers are aware that their smoking causes some amount of harm.[10] However, recent studies have found that some adult smokers perceive the health risks of LCC smoking to be minimal, noting that any cigar use (including LCCs) is less harmful than cigarette smoking.[11–14] In addition to the availability flavor additives in LCC tobacco, widespread product advertising may also contribute to these misperceptions. The text, imagery, and color of LCC advertisements and packaging are designed to convey the sense of a lighter and perhaps healthier product than cigarettes.[15–17] Adults may endorse the perceived safety of LCCs because, unlike cigarettes, they are not addressed in anti-smoking media campaigns.[18–20] Further, infrequent non-daily use[14,21] and misperceptions about the constituents of LCCs, such as the belief that LCCs have "natural" or fewer harmful ingredients[18,20,21] compared to cigarettes, may also underscore these perceptions of reduced risk.

Despite well-established associations between risk perceptions and smoking behavior,[22] few have examined the perceptions of risk of LCC smoking among adult cigarette smokers. The current study sought to understand adult current cigarette smokers' perceptions of harm related to LCC smoking. We also assessed LCC smoking behavior outcomes, including LCC smoking experimentation (ever use), susceptibility to LCC use, and intention to continue using LCCs, among our respondents. The association among perceptions of risk and the outcomes susceptibility to use and intention to continue smoking LCCs were also assessed. Understanding cigarette smokers' perceptions of risk and its association with LCC smoking behaviors will contribute to the body of evidence that can be used to expand future FDA regulatory policies related to LCCs. Study data can also contribute to the development of programs, including health communication campaigns that seek to curtail LCC use among cigarette smokers.

Methods

Study Overview and Participants

Data for the current study come from a larger study conducted by the Georgia State University Tobacco Center of Regulatory Sciences (TCORS) that seeks to understand consumers' perceptions of risk about novel and alternative tobacco products. In 2014, the parent study administered a cross-sectional survey, the Tobacco Products and Perceptions Survey, to a probability web-based sample of non-institutionalized US adults, aged 18 and older. The sample was drawn from GfK's KnowledgePanel, an online research panel, and included a representative oversample of cigarette smokers. We invited 7991 KnowledgePanel members to participate in the survey. Of those, 5833 completed the survey; 116 respondents were excluded because they did not complete more than 50% of the survey items. A total of 5717 respondents were retained for analyses. Additional details about the parent study can be found elsewhere.[23] This study was approved by the Institutional Review Board of Georgia State University.

Measures

Measures assessed in the current study included respondents' sociodemographic characteristics, cigarette and LCC smoking status, and perceptions of risks about LCC use. Sociodemographic characteristics assessed included sex, age, race/ethnicity, education, annual household income, and employment status. Cigarette smoking status was assessed with two items: lifetime smoking of at least 100 cigarettes (response options: yes/no) and currently smoking cigarettes "every day," "some days," or "not at all." Respondents who reported smoking at least 100 cigarettes in their lifetime and smoked cigarettes "every day" or "some days" were classified as current cigarette smokers. Current cigarette smokers were asked about their LCC smoking status. LCC smoking status was assessed by asking respondents about ever use of LCCs (response options: yes/no). Brand names and product images were included with a definition of LCCs to assist respondents with product recognition. Current cigarette smokers who reported ever trying a LCC were asked about their intention to smoke LCCs again in the future. Intention to smoke LCCs in the future was assessed by the following question: "Which of the following best describes your thoughts on smoking little cigars or cigarillos in the future?" Response options included "probably won't smoke LCCs again," "probably will smoke LCCs for a short time"; "probably will smoke LCCs for a long time"; and "don't know." Current cigarette smokers who had never tried smoking a LCC were asked about their susceptibility to use LCCs. Susceptibility to use LCCs was assessed with the following question: "How likely are you to try smoking little cigars or cigarillos in the next year?" (response options: "very unlikely" to "very likely"). Current cigarette smokers who said they would "very likely" try LCCs were defined as LCC-susceptible cigarette smokers, while those who said "very unlikely" were defined as non-LCC-susceptible cigarette smokers. Perception of risk of LCC use was assessed by asking respondents about the likelihood of becoming addicted to LCCs (response options: "yes," "no," "don't know"); the harmfulness of smoking LCCs compared to cigarette smoking (response options: "less harmful," "more harmful," "about the same harm," and "don't know"); risk of smoking LCCs daily (response options: "very risky" to "not risky at all," and "don't know"); and risk of smoking LCCs occasionally (response options: "very risky" to "not risky at all," and "don't know").

Analytic Sample

Of the 5717 respondents, 1349 were current cigarette smokers; 158 respondents did not provide data on their LCC smoking status and were excluded from the analyses. Data from the 158 respondents were missing at random and did not influence study results. A total of 1191 current cigarette smokers were stratified into three groups: (1) dual current cigarette smokers who had ever smoked LCCs (Group 1); (2) LCC-susceptible cigarette smokers (Group 2); and (3) non-LCC-susceptible cigarette smokers (Group 3). Ever LCC smoking (instead of current smoking) was used to define dual use, as the sample size for current (past 30-day) LCC smokers was too small (n = 92) to produce reliable estimates. Of 1191 respondents, 49.1% (n = 584) were dual cigarette and ever LCC smokers, 9.8% (n = 117) were LCC-susceptible cigarette smokers, and 41.1% (n = 490) were non-LCC-susceptible cigarette smokers.

Analyses

Weighted descriptive and inferential statistics were conducted using Stata 14.0. Descriptive statistics were used to describe the sociodemographic characteristics of the 1191 cigarette smoking respondents. Multinomial logistic regression analyses were conducted to compare the sociodemographic factors and risk perception items among the three subgroups. Multinomial logistic regression analyses were also conducted to examine the association among the risk perception items and intention to smoke LCCs in the future among dual smokers (Group 1). Finally, multivariate logistic regression analyses were conducted to examine the association among the risk perception items and susceptibility to use LCCs among cigarette smokers who did not have a history of LCC use (Groups 2 and 3 combined). All multinomial and multivariate models were controlled for sociodemographic factors to produce adjusted relative risk ratios (RRR).

Results

Sociodemographic Characteristics of the Total Sample and Smoking Status Subgroups

Respondents' sociodemographic and smoking characteristics are presented in Table 1. Overall, the majority of respondents were white, male, and aged 45–59 years old. Table 1 also presents the results of the multinomial logistic regression analyses that compared the sociodemographic factors between dual smokers and non-LCC-susceptible cigarette smokers (Group 1 vs. Group 3) and LCC-susceptible smokers and non-LCC-susceptible cigarette smokers (Group 2 vs. Group 3). Compared to males, female cigarette smokers had lower odds of dual smoking (Group 1) than non-LCC-susceptible cigarette smoking (Group 3). Compared to adults aged 18–29 years old, cigarette smoking adults aged 45–59 years old and those age 60 years and older had lower odds of dual smoking (Group 1) than non-LCC-susceptible cigarette smoking (Group 3).

Compared to adults aged 18–29 years old, adults aged 45–59 years old also had lower odds of LCC-susceptible smoking (Group 2) than non-LCC-susceptible smoking (Group 3). Compared to white, non-Hispanics, black, non-Hispanics, and other, non-Hispanics had greater odds of LCC-susceptible cigarette smoking (Group 2) than non-LCC-susceptible cigarette smoking (Group 3).

Perceptions of Risk of LCCs by Smoking Status Subgroups

As shown in Table 2, the majority of the 1191 respondents believed that LCCs were addictive. Over half of the respondents believed that smoking LCCs was just as harmful as cigarette smoking. With regard to frequency of use, 42.4% of respondents believed that daily LCC smoking was "very risky," while only 22.1% of respondents believed that occasional LCC smoking was "very risky".

When comparing risk perception items across the smoking subgroups, the multinomial logistic regression analyses found that, compared to cigarette smokers who believed that daily LCC smoking was "very risky," those who believe it was "somewhat risky" had greater odds of dual smoking; those who were uncertain about the risk of daily LCC smoking had lower odds of dual smoking (Group 1) than non-LCC-susceptible cigarette smoking (Group 3). Further, compared to cigarette smokers who believed that occasional LCC smoking was "very risky," those who believed that occasional use was "a little risky" had greater odds of dual smoking (Group 1) than non-LCC-susceptible cigarette smoking (Group 3).

Compared to those who believed that LCCs were addictive, those who were uncertain about their addictiveness had greater odds of LCC-susceptible smoking (Group 2) than non-LCC-susceptible smoking (Group 3). Compared to those who believed that daily LCC smoking was "very risky", those who believed that daily use was "a little risky" had greater odds of LCC-susceptible smoking (Group 2) than non-LCC-susceptible smoking (Group 3).

Risk Perceptions and Intention to Smoke LCCs in the Future

Among the 584 dual smokers, 52% indicated that they did not intend to smoke LCCs again; 20.7% intended to smoke LCCs for a short or long time; and 27.3% were unsure if they would smoke LCCs again. Table 3 shows the association between the risk perception items and dual smokers' intent to smoke LCCs again in the future. Compared to smokers who said that LCCs were addictive, those who believed that LCCs were not addictive had 4.8 times the odds of intending to smoke LCCs again compared to never smoking LCCs again. Compared to smokers who believed that smoking LCCs daily was "very risky", those who believed that daily LCC use was "somewhat risky" had 2.3 times the odds of intending to smoke LCCs again compared to never smoking LCCs again. Notably, cigarette smokers who believed that daily LCC use was "somewhat risky" also had greater odds of being uncertain about their intention to smoke LCCs again in the future, compared to never smoking LCCs again. Finally, those who were uncertain about the harmfulness of daily LCC smoking had 6.8 times the odds of intending to smoke LCCs again compared to never smoking LCCs again. Cigarette smokers who were uncertain about the harmfulness of daily LCC smoking also had almost 13 times the odds of being uncertain about their intention to smoke LCCs in the future, compared to never smoking LCCs again.

Risk Perceptions and Susceptibility to Use LCCs

We conducted a multivariate logistic regression analysis, controlling for sociodemographic factors, to examine the association between perceived risk and susceptibility to use LCCs among cigarette smokers who did not have a history of LCC smoking (Groups 2 and 3 combined). As shown in Table 4, only perceptions about the addictiveness of LCC smoking predicted susceptibility to use LCCs. Compared to those who said that LCCs are addictive, those who were uncertain about the addictiveness of LCCs were more likely to be susceptible to smoking the product in the future.

Discussion

Our study documents the association between perceptions of risk about LCC smoking and the outcomes dual LCC use, susceptibility to use LCCs, and intention to continue LCC use among a probability

Table 1. Sociodemographic Characteristics and Smoking Status Among US Current Cigarette Smokers

Sociodemographic characteristics	Smoking status					
	Overall (N = 1191)	Group 1: Dual smokers (n = 584)	Group 2: LCC-susceptible cigarette smokers (n = 117)	Group 3: Non-LCC-susceptible cigarette smokers (n = 490)	Group 1 vs. Group 3	Group 2 vs. Group 3
	%	%	%	%	Adjusted RRR (95% CI)	Adjusted RRR (95% CI)
Prevalence		47.2	12.7	40.1		
Sex						
Male	52.5	65.4	47.6	38.8	Ref.	Ref.
Female	47.5	34.6	52.4	61.2	0.337** (0.248–0.459)	0.677 (0.415–1.107)
Age						
18–29	21.7	24.5	34.6	14.4	Ref.	Ref.
30–44	28.7	30.1	33.6	25.7	0.705 (0.417–1.194)	0.498 (0.248–1.001)
45–59	31.9	29.9	27.3	35.6	0.543* (0.331–0.889)	0.313** (0.160–0.612)
≥60	17.7	15.6	4.4	24.4	0.371** (0.220–0.627)	0.0793** (0.0300–0.210)
Race/ethnicity						
White, non-Hispanic	63.2	69.1	44.0	62.4	Ref.	Ref.
Black, non-Hispanic	17.2	14.2	26.9	17.7	0.789 (0.504–1.235)	2.019* (1.061–3.840)
Other, non-Hispanic	6.4	5.1	14.7	5.4	0.692 (0.366–1.310)	3.391** (1.483–7.755)
Hispanic	13.1	11.6	14.3	14.5	0.624 (0.362–1.075)	1.043 (0.488–2.225)
Education						
Less than high school	22.2	20.1	32.1	21.6	Ref.	Ref.
High school	36.1	33.8	29.3	41.0	0.812 (0.502–1.313)	0.557 (0.282–1.102)
Some college	31.0	33.3	30.7	28.5	1.167 (0.731–1.864)	0.925 (0.474–1.802)
Bachelor's degree +	10.6	12.8	7.9	9.0	1.400 (0.777–2.523)	0.681 (0.243–1.908)
Income						
<$15 000	24.8	22.1	34.4	25.1	Ref.	Ref.
$15 000 to $24 999	10.8	9.1	15.4	11.4	0.801 (0.444–1.443)	1.200 (0.505–2.852)
$25 000 to $39 999	20.2	19.7	18.2	21.3	1.059 (0.641–1.748)	0.775 (0.370–1.622)
$40 000 to $59 999	17.4	17.4	13.3	18.7	0.880 (0.539–1.438)	0.627 (0.286–1.375)
$60 000 to $84 999	11.9	15.6	5.9	9.4	1.440 (0.817–2.539)	0.502 (0.183–1.378)
$85 000 to $99 999	5.0	6.2	1.8	4.6	1.092 (0.518–2.304)	0.288 (0.0639–1.297)
>$100 000	9.8	9.8	10.9	9.5	0.849 (0.455–1.582)	0.958 (0.381–2.407)
Employment						
Not employed	49.8	46.3	53.9	52.6	Ref.	Ref.
Employed	50.2	53.7	46.1	47.4	0.923 (0.663–1.285)	1.028 (0.569–1.858)

CI = confidence interval; LCC = little cigar and cigarillo; RRR = Relative risk ratio. All % are column%.
*p < .05; **p < .01; ***p < .001.

Table 2. Perceived Risk of LCCs by Smoking Status Among US Current Cigarette Smokers

	Overall (N = 1191)	Group 1: Dual smokers (n = 584)	Group 2: LCC-susceptible cigarette smokers (n = 117)	Group 3: Non-LCC-susceptible cigarette smokers (n = 490)	Group 1 vs. Group 3	Group 2 vs. Group 3
Risk Perceptions	%	%	%	%	Adjusted RRR (95% CI)	Adjusted RRR (95% CI)
Become addicted to LCCs?						
No	4.6	5.5	7.2	2.8	Ref.	Ref.
Yes	73.1	76.2	52.1	76.1	1.720 (0.739–4.003)	2.448 (0.818–7.325)
I don't know	22.3	18.3	40.7	21.1	1.048 (0.670–1.639)	2.829** (1.571–5.094)
Harmfulness of LCC smoking compared to cigarettes						
Less harmful	3.1	4.3	2.8	1.7	Ref.	Ref.
About the same	58.6	58.0	55.4	60.3	1.745 (0.680–4.476)	0.851 (0.198–3.658)
More harmful	13.9	16.9	8.2	12.1	1.533 (0.968–2.426)	0.814 (0.371–1.783)
I don't know	24.4	20.8	33.6	25.9	1.318 (0.814–2.133)	0.956 (0.451–2.027)
Harmfulness of smoking LCCs daily						
Very risky	42.4	42.6	26.8	46.9	Ref	Ref
Somewhat risky	26.0	32.4	22.9	19.4	1.614* (1.083–2.405)	1.875 (0.880–3.994)
A little risky	8.6	8.3	18.3	6.1	0.915 (0.451–1.856)	3.136* (1.123–8.759)
Not at all risky	0.6	0.7	1.2	0.0	0.816 (0.105–6.296)	2.495 (0.265–23.457)
I don't know	22.3	15.9	30.8	27.2	0.426* (0.211–0.857)	1.349 (0.488–3.727)
Harmfulness of smoking LCCs occasionally						
Very risky	22.1	19.7	16.4	26.8	Ref	Ref
Somewhat risky	29.3	31.4	25.7	28.0	1.339 (0.861–2.082)	1.182 (0.521–2.680)
A little risky	20.3	24.6	18.4	15.8	1.710* (1.019–2.870)	1.059 (0.420–2.670)
Not at all risky	4.7	5.5	7.7	2.8	2.277 (0.957–5.414)	2.324 (0.631–8.550)
I don't know	23.6	18.8	31.8	26.6	1.681 (0.801–3.524)	0.839 (0.292–2.408)

CI = confidence interval; LCC = little cigar and cigarillo; RRR = Relative risk ratio. All % are column%. Multinomial logistic regression models in this table were conducted with all variables shown and controlled for sociodemographic factors.

*p < .05; **p < .01; ***p < .001.

Table 3. Predictors of Intention to Continue to Smoke LCCs in the Future Among US Dual Current Cigarette Smokers and Ever LCC Users ($n = 584$)

	"Will smoke LCC again" vs. "Won't smoke LCC again"	"I don't know" vs. "Won't smoke LCC again"
Risk perception	Adjusted RRR (95% CI)	Adjusted RRR (95% CI)
Become addicted to LCC?		
Yes	Ref.	Ref.
No	4.831 (1.148–20.34)*	2.662 (0.652–10.86)
I don't know	1.662 (0.795–3.475)	0.910 (0.415–1.993)
Harmfulness of LCC smoking compared to cigarette smoking		
About the same level of harm	Ref.	Ref.
Less harmful	1.000 (0.313–3.199)	0.267 (0.0716–0.994)*
More harmful	0.875 (0.395–1.937)	0.431 (0.191–0.974)*
I don't know	0.479 (0.208–1.102)	0.702 (0.292–1.687)
Harmfulness of smoking LCCs daily		
Very risky	Ref.	Ref.
Somewhat risky	2.300 (1.102–4.802)*	2.595 (1.331–5.063)**
A little risky	3.243 (0.990–10.62)	4.850 (1.877–12.53)**
Not at all risky	0.472 (0.0576–3.866)	—
I don't know	6.788 (1.555–29.64)*	12.95 (3.956–42.39)***
Harmfulness of smoking LCCs occasionally		
Very risky	Ref.	Ref.
Somewhat risky	1.019 (0.441–2.354)	0.892 (0.405–1.964)
A little risky	0.646 (0.237–1.760)	0.508 (0.217–1.189)
Not at all risky	0.656 (0.144–2.986)	0.543 (0.135–2.175)
I don't know	0.366 (0.0900–1.491)	0.602 (0.195–1.859)

CI = confidence interval; LCC = little cigar and cigarillo; RRR = Relative risk ratio. Multinomial logistic regression models in this table were conducted with all variables shown and controlled for sociodemographic factors.
*$p < .05$; **$p < .01$; ***$p < .001$.

Table 4. Predictors of Susceptibility to Smoke LCCs Among US Current Cigarette Smokers Who Have not Used LCCs ($n = 607$)

	Susceptibility to smoke LCCs
Risk perception	*AOR* (95% CI)
Become addicted to LCCs?	
Yes	Ref.
No	3.170 (0.948–10.60)
I don't know	2.908** (1.555–5.437)
Harmfulness of LCC smoking compared to cigarette smoking	
About the same	Ref.
Less harmful	0.866 (0.206–3.639)
More harmful	0.755 (0.332–1.721)
I don't know	1.176 (0.512–2.700)
Harmfulness of smoking LCCs daily	
Very risky	Ref.
Somewhat risky	1.980 (0.892–4.396)
A little risky	2.938 (0.959–9.005)
Not at all risky	1.299 (0.0869–19.41)
I don't know	1.209 (0.371–3.939)
Harmfulness of smoking LCCs occasionally	
Very risky	Ref.
Somewhat risky	1.083 (0.446–2.626)
A little risky	0.810 (0.294–2.233)
Not at all risky	1.570 (0.424–5.818)
I don't know	0.655 (0.192–2.234)

AOR = Adjusted Odds Ratio; CI = confidence interval; LCC = little cigar and cigarillo. Multivariable logistic regression model in this table was conducted with all variables shown and controlled for sociodemographic factors.
*$p < .05$; **$p < .01$.

sample of US adult cigarette smokers. Overall, the majority of smokers in our sample perceived that LCCs were addictive and that smoking LCCs carries the same level of harm as cigarettes. With regard to frequency of use, the majority of respondents perceived daily LCC use to be more harmful than occasional use. Differences in perceptions about the frequency of LCC use were also found across the smoking status subgroups. Compared to those not susceptible to LCC smoking, dual smokers were more likely to perceive that daily LCC use was "somewhat" risky while occasional LCC smoking was "a little" risky; LCC-susceptible smokers also perceived daily LCC smoking to be "a little risky." Prior studies have found that, unlike cigarette smokers, smokers who use LCCs typically smoke the product less frequently and do not smoke a whole LCC at once.[11,14] All cigar smoking, including infrequent use, produces toxic smoke[24,25] and can lead to the absorption of nicotine and other harmful constituents[26,27] that may increase the risk of several types of chronic diseases and other adverse health affects.[26–28] Though respondents' perceptions are inaccurate, it is possible that LCC smoking behavior patterns (ie, smoking them less frequently) contribute to perceptions about health risks.

Our findings suggest that perceptions of risk were not merely subjective beliefs but were also important determinants of LCC smoking behavior. Compared to cigarette smokers who were not susceptible to LCC use, dual smokers who believed that daily use carried minimal risk and believed that LCCs were not addictive were more likely to intend to smoke the product again in the future. As documented in prior studies, some cigarette smokers presume that all cigars (including LCCs) are less dangerous than and are safer alternatives to cigarettes.[13,29] That these misperceptions are associated with continued LCC smoking among dual smokers is troubling;

dual cigarette and LCC use may increase the likelihood of nicotine dependence,[11,30] cancer,[25,26,31] and other chronic health conditions.[25,32]

Equally concerning was the degree of uncertainty about the addictiveness of LCC smoking among LCC-susceptible cigarette smokers. Over 40% of LCC-susceptible cigarette smokers were uncertain about its addictiveness, and those who were uncertain were almost three times more likely to be susceptible to trying LCC smoking. Richter and colleagues[19] found that a lack of coverage of cigars in anti-tobacco health education campaigns explained perceptions of reduced risk for cigar smoking. Continued lack of coverage may be fueling the uncertainty of and misperceptions about risk for cigar smoking among these smokers. The FDA now requires warning labels to be placed on cigar packages[33]; this is an important step to correct misperceptions of the addictiveness and safety of cigars.

The study is not without limitations. First, our measures of risk perception were limited, and only assessed perceived addictiveness of LCCs; its harm compared to cigarette smoking; and harm associated with daily and occasional use of LCCs. Other published studies suggest that perceptions of risk about LCCs are shaped by numerous factors, including, but not limited to, consumers' beliefs about the products' constituents, the flavored tobacco in the LCCs, and LCC package descriptors (eg, text, color).[12,14,19] Additional studies using expanded measures of perceptions of risk are needed. Second, our definition of dual smokers included ever (rather than current) LCC smoking. Future studies that define dual smokers as current (ie, past 30-day) cigarette and LCC users are warranted to replicate our study findings. We assessed susceptibility to trying LCCs using a single questionnaire item, rather than the traditional three to four items that have been validated for cigarette smoking.[34] As such, study findings should be interpreted with caution.

Our study suggests that perceptions of risk about the addictiveness of LCCs and frequency of LCC use are important determinants of intention to continue LCC use among cigarette smokers with a history of LCC use. These perceptions are also determinants of LCC smoking susceptibility among cigarette smokers. The Center for Tobacco Products is tasked with correcting misperceptions about regulated tobacco products and educating the public about the dangers of use, including health risks and addictiveness of the product. As noted above, the Center for Tobacco Products will require advertising and package warning labels, including addictiveness warning labels, for all newly deemed products, including LCCs.[33] Once the warnings are implemented, future studies should evaluate the effectiveness of the warning labels on adult smokers' perceptions of the addictiveness of LCCs to further inform the development of health communication campaigns. Misperceptions about the frequency of LCC use and its association with LCC smoking outcomes (ie, intention to continue use) suggest the need for additional health communication messages that convey that any use of cigars (either daily or occasional use) is harmful and carries the risk of adverse negative health outcomes. Current FDA initiatives, that is, the Fresh Empire campaign,[35] may be appropriate to address these misperceptions among adults.

Funding

This work was supported by the National Institutes of Health, National Institute of Drug Abuse (NIH/NIDA) and Food and Drug Administration, Center for Tobacco Products (FDA CTP) (P50DA036128, ME) and National Institutes of Health, National Cancer Institute (NIH/NCI) and Food and Drug Administration, Center for Tobacco Products (FDA CTP) (1R21CA180934-01, KLS).

Declaration of Interests

None declared.

References

1. Agaku IT, King BA, Husten CG, et al. Tobacco product use among adults--United States, 2012–2013. *MMWR Morb Mortal Wkly Rep*. 2014;63(25):542–547.
2. Delnevo CD, Giovenco DP, Ambrose BK, Corey CG, Conway KP. Preference for flavoured cigar brands among youth, young adults and adults in the USA. *Tob Control*. 2015;24(4):89–94. doi:10.1136/tobaccocontrol-2013-051408.
3. Cantrell J, Kreslake JM, Ganz O, et al. Marketing little cigars and cigarillos: advertising, price, and associations with neighborhood demographics. *Am J Public Health*. 2013;103(10):1902–1909. doi:10.2105/AJPH.2013.301362.
4. Cohn A, Cobb CO, Niaura RS, Richardson A. The other combustible products: prevalence and correlates of little cigar/cigarillo use among cigarette smokers. *Nicotine Tob Res*. 2015;17(12):1473–1481. doi:10.1093/ntr/ntv022.
5. Rath JM, Villanti AC, Abrams DB, Vallone DM. Patterns of tobacco use and dual use in US young adults: the missing link between youth prevention and adult cessation. *J Environ Public Health*. 2012;2012:1–9. doi:10.1155/2012/679134.
6. Richardson A, Xiao H, Vallone DM. Primary and dual users of cigars and cigarettes: profiles, tobacco use patterns and relevance to policy. *Nicotine Tob Res*. 2012;14(8):927–932. doi:10.1093/ntr/ntr306.
7. Corey CG, King BA, Coleman BN, et al. Little filtered cigar, cigarillo, and premium cigar smoking among adults--United States, 2012–2013. *MMWR Morb Mortal Wkly Rep*. 2014;63(30):650–654.
8. Messer K, White MM, Strong DR, et al. Trends in use of little cigars or cigarillos and cigarettes among U.S. smokers, 2002–2011. *Nicotine Tob Res*. 2015;17(5):515–523. doi:10.1093/ntr/ntu179.
9. King BA, Dube SR, Tynan MA. Flavored cigar smoking among U.S. adults: findings from the 2009–2010 National Adult Tobacco Survey. *Nicotine Tob Res Off J Soc Res Nicotine Tob*. 2013;15(2):608–614. doi:10.1093/ntr/nts178.
10. Wray RJ, Jupka K, Berman S, Zellin S, Vijaykumar S. Young adults' perceptions about established and emerging tobacco products: results from eight focus groups. *Nicotine Tob Res*. 2012;14(2):184–190. doi:10.1093/ntr/ntr168.
11. Baker F, Dye JT, Denniston MM, Ainsworth SR. Risk perception and cigar smoking behavior. *Am J Health Behav*. 2001;25(2):106.
12. Malone RE, Yerger V, Pearson C. Cigar risk perceptions in focus groups of urban African American youth. *J Subst Abuse*. 2001;13(4):549–561.
13. Nyman AL, Taylor TM, Biener L. Trends in cigar smoking and perceptions of health risks among Massachusetts adults. *Tob Control*. 2002;11(suppl 2):ii25–28.
14. Sterling KL, Fryer CS, Fagan P. The most natural tobacco used: a qualitative investigation of young adult smokers' risk perceptions of flavored little cigars and cigarillos. *Nicotine Tob Res*. 2016;18(5):827–833. doi:10.1093/ntr/ntv151.
15. National Cancer Institute. *The Role of the Media in Promoting and Reducing Tobacco Use. Tobacco Control Monograph No. 19*. Bethesda, MD: U.S. Department of Health and Human Services, National Institutes of Health, National Cancer Institute; 2008.
16. Etzel E, Monohan E, Ece I. Camel filter revised packaging test: Consumer research proposal. 1979. http://legacy.library.ucsf.edu/tid/qxb79d00. Accessed September 8, 2016.
17. Gauvin G. *Putting an End to Deception: A Report to the Canadian Minister of Health*. Ottawa, ON, Canada: Ministerial Advisory Council on Tobacco Control; 2001. www.cctc.ca/cctc/EN/fundamentals/ cessation/basics/Putting_an_End_to_Deception.pdf/view. Accessed September 8, 2016.
18. Jolly DH. Exploring the use of little cigars by students at a historically black university. *Prev Chronic Dis*. 2008;5(3):A82.

19. Richter P, Caraballo R, Pederson LL, Gupta N. Exploring use of nontraditional tobacco products through focus groups with young adult smokers, 2002. *Prev Chronic Dis*. 2008;5(3):A87.
20. Page JB, Evans S. Cigars, cigarillos, and youth -- emergent patterns in subcultural complexes. *J Ethn Subst Abuse*. 2004;2(4):63. doi:10.1300/J233v02n04_04.
21. Richter PA, Pederson LL, O'Hegarty MM. Young adult smoker risk perceptions of traditional cigarettes and nontraditional tobacco products. *Am J Health Behav*. 2006;30(3):302–312. doi:10.5555/ajhb.2006.30.3.302.
22. Slovic P. *Smoking Risk, Perception & Policy*. Thousand Oaks, CA: Sage Publications; 2001.
23. Weaver SR, Majeed BA, Pechacek TF, Nyman AL, Gregory KR, Eriksen MP. Use of electronic nicotine delivery systems and other tobacco products among USA adults, 2014: results from a national survey. *Int J Public Health*. 2016;61(2):177–188. doi:10.1007/s00038-015-0761-0.
24. Baker F, Ainsworth SR, Dye JT, Crammer C, Thun M, Hoffmann D. Health risks associated with cigar smoking. *J Am Med Assoc*. 2000;284(18):735–740.
25. National Cancer Institute. *Cigars: Health Effects and Trends. Smoking and Tobacco Control Monograph No. 9*. Bethesda, MD: U.S. DHHS, National Institutes of Health, National Cancer Institute; 1998.
26. Rodriguez J, Jiang R, Johnson WC, MacKenzie BA, Smith LJ, Barr RG. The association of pipe and cigar use with cotinine levels, lung function, and airflow obstruction: a cross-sectional study. *Ann Intern Med*. 2010;152(4):201–210. doi:10.1059/0003-4819-152-4-201002160-00004.
27. Chen J, Kettermann A, Rostron BL, Day HR. Biomarkers of exposure among U.S. cigar smokers: an analysis of 1999–2012 National Health and Nutrition Examination Survey (NHANES) data. *Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol*. 2014;23(12):2906–2915. doi:10.1158/1055-9965.EPI-14-0849.
28. Wyss A, Hashibe M, Chuang S-C, et al. Cigarette, cigar, and pipe smoking and the risk of head and neck cancers: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Am J Epidemiol*. 2013;178(5):679–690. doi:10.1093/aje/kwt029.
29. Latimer LA, Batanova M, Loukas A. Prevalence and harm perceptions of various tobacco products among college students. *Nicotine Tob Res*. 2014;16(5):519–526. doi:10.1093/ntr/ntt174.
30. Hoffmann D, Wynder EL. Smoke of cigarettes and little cigars: an analytical comparison. *Science*. 1972;178(4066):1197–1199.
31. Boffetta P, Nyberg F, Agudo A, et al. Risk of lung cancer from exposure to environmental tobacco smoke from cigars, cigarillos and pipes. *Int J Cancer J Int Cancer*. 1999;83(6):805–806.
32. Iribarren C, Tekawa IS, Sidney S, Friedman GD. Effect of cigar smoking on the risk of cardiovascular disease, chronic obstructive pulmonary disease, and cancer in men. *N Engl J Med*. 1999;340(23):1773–1780. doi:10.1056/NEJM199906103402301.
33. Pierce JP, Choi WS, Gilpin EA, Farkas AJ, Merritt RK. Validation of susceptibility as a predictor of which adolescents take up smoking in the United States. *Health Psychol*. 1996;15(5):355–361.
34. U. S. Food and Drug Administration. Deeming Tobacco Products To Be Subject to the Federal Food, Drug, and Cosmetic Act, as Amended by the Family Smoking Prevention and Tobacco Control Act; Restrictions on the Sale and Distribution of Tobacco Products and Required Warning Statements for Tobacco Products. 2016:1–499. www.federalregister.gov/articles/2016/05/10/2016–10685/deeming-tobacco-products-to-be-subject-to-the-federal-food-drug-and-cosmetic-act-as-amended-by-the. Accessed May 10, 2016.
35. Food and Drug Administration, Center for Tobacco Products. Fresh Empire Campaign. 2015. www.fda.gov/TobaccoProducts/PublicHealthEducation/PublicEducationCampaigns/FreshEmpireCampaign/default.htm. Accessed June 22, 2016.

Centers for Disease Control and Prevention
MMWR
Morbidity and Mortality Weekly Report
Weekly / Vol. 61 / No. 30
August 3, 2012

Consumption of Cigarettes and Combustible Tobacco — United States, 2000–2011

Smoking cigarettes and other combustible tobacco products causes adverse health outcomes, particularly cancer and cardiovascular and pulmonary diseases (*1*). A priority of the U.S. Department of Health and Human Services is to develop innovative, rapid-response surveillance systems for assessing changes in tobacco use and related health outcomes (*2*). The two standard approaches for measuring smoking rates and behaviors are 1) surveying a representative sample of the public and asking questions about personal smoking behaviors and 2) estimating consumption based on tobacco excise tax data (*3*). Whereas CDC regularly publishes findings on national and state-specific smoking rates from public surveys (*4*), CDC has not reported consumption estimates. The U.S. Department of Agriculture (USDA), which previously provided such estimates, stopped reporting on consumption in 2007 (*5*). To estimate consumption for the period 2000–2011, CDC examined excise tax data from the U.S. Department of Treasury's Alcohol and Tobacco Tax and Trade Bureau (TTB); consumption estimates were calculated for cigarettes, roll-your-own tobacco, pipe tobacco, and small and large cigars. From 2000 to 2011, total consumption of all combustible tobacco decreased from 450.7 billion cigarette equivalents to 326.6, a 27.5% decrease; per capita consumption of all combustible tobacco products declined from 2,148 to 1,374, a 36.0% decrease. However, while consumption of cigarettes decreased 32.8% from 2000 to 2011, consumption of loose tobacco and cigars increased 123.1% over the same period. As a result, the percentage of total combustible tobacco consumption composed of loose tobacco and cigars increased from 3.4% in 2000 to 10.4% in 2011. The data suggest that certain smokers have switched from cigarettes to other combustible tobacco products, most notably since a 2009 increase in the federal tobacco excise tax that created tax disparities between product types.

USDA's previous consumption estimates were based on 1) information from TTB, including data on products that are produced domestically or imported and taxed for legal sale in the United States; 2) tobacco industry reports; and 3) information from industry advisors. CDC developed a method to estimate consumption exclusively by using publicly available federal excise tax data available from TTB on products taxed domestically and imported into the United States (*6*). Using monthly tax data, CDC calculated the per unit (e.g., per cigarette or per cigar) consumption for each product. To enable comparisons with pipe tobacco and roll-your-own tobacco, CDC converted the tax data from pounds of tobacco to a per cigarette equivalent, based on the conversion formula contained in the Master Settlement Agreement (0.0325 oz [0.9 g] = one cigarette).* Adult per capita cigarette consumption was estimated by dividing total consumption by the number of persons aged ≥18 years in the United States each year using data from the U.S. Census Bureau. When compared with USDA's previous calculations for adult per capita cigarette consumption during 2000–2006, CDC's estimates differed each year by a median of only 0.15% and a mean of 0.76%.

From 2000 to 2011, total cigarette consumption declined from 435.6 billion to 292.8 billion, a 32.8% decrease (Table 1). Per capita cigarette consumption declined from 2,076 in 2000 to 1,232 in 2011, a 40.7% decrease. Conversely, total consumption of noncigarette combustible products increased

* Available at http://www.naag.org/backpages/naag/tobacco/msa/msa-pdf.

Continuing Education examination available at http://www.cdc.gov/mmwr/cme/conted_info.html#weekly.

U.S. Department of Health and Human Services
Centers for Disease Control and Prevention

from 15.2 billion cigarette equivalents in 2000 to 33.8 billion in 2011, a 123.1% increase, and per capita consumption increased from 72 in 2000 to 142 in 2011, a 96.9% increase. Total consumption of all combustible tobacco decreased from 450.7 billion cigarette equivalents to 326.6, a 27.5% decrease from 2000 to 2011, and per capita consumption of all combustible tobacco products declined from 2,148 to 1,374, a 36.0% decrease.

Consumption of loose tobacco (i.e., roll-your-own cigarette tobacco and pipe tobacco) changed substantially from 2000 to 2011. Roll-your-own cigarette equivalent consumption decreased by 56.3%, whereas pipe tobacco consumption increased by 482.1% (Table 2). The largest changes occurred from 2008 to 2011, when roll-your-own consumption decreased from 10.7 billion to 2.6 billion (a 75.7% decrease), whereas pipe tobacco consumption increased from 2.6 billion to 17.5 billion (a 573.1% increase).

Substantial changes also were observed in consumption of small cigars[†] and large cigars (Figure 1). From 2000 to 2011, consumption of small cigars decreased 65.0%, whereas large cigar consumption increased 233.1% (Table 2). The largest changes occurred from 2008 to 2011, when small cigar consumption decreased from 5.9 billion to 0.8 billion (an 86.4% decrease), whereas large cigar consumption increased from 5.7 billion to 12.9 billion (a 126.3% increase).

[†] In 26 USC 5701, small cigars are defined as cigars that weigh ≥3 pounds (<1.36 kg) per 1,000 cigars, and large cigars are defined as cigars that weigh >3 pounds per 1,000.

Annual cigarette consumption declined each year during 2000–2011, including a 2.6% decrease from 2010 to 2011, but total consumption of combustible tobacco decreased only 0.8% from 2010 to 2011, in part because of the effect of continued increases in the consumption of noncigarette combustible tobacco products (Figure 2). From 2000 to 2011, the percentage of total combustible tobacco consumption composed of loose tobacco and cigars increased from 3.4% (15.2 billion cigarette equivalents out of 450.7 billion) to 10.4% (33.8 billion of 326.6 billion).

Reported by

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Editorial Note

Despite continued decreases in cigarette smoking in the United States, consumption of pipe tobacco and large cigars has increased substantially since the federal tobacco excise tax was increased in 2009, creating tax disparities that made 1) pipe tobacco less expensive than roll-your-own tobacco and manufactured cigarettes, and 2) large cigars less heavily taxed than small cigars and manufactured cigarettes (*7,8*). Because loose tobacco products are classified based on how they are labeled, the loose tobacco tax disparity of $21.95 per pound

The *MMWR* series of publications is published by the Office of Surveillance, Epidemiology, and Laboratory Services, Centers for Disease Control and Prevention (CDC), U.S. Department of Health and Human Services, Atlanta, GA 30333.

Suggested citation: Centers for Disease Control and Prevention. [Article title]. MMWR 2012;61:[inclusive page numbers].

TABLE 1. Total consumption and adult per capita consumption* of cigarettes, all combustible tobacco,† and noncigarette combustible tobacco products§ — United States, 2000–2011

Year	Cigarettes				All combustible tobacco				Noncigarette combustible tobacco			
	Total consumption (in millions)	% change	Adult per capita consumption	% change	Total consumption (in millions)	% change	Adult per capita consumption	% change	Total consumption (in millions)	% change	Adult per capita consumption	% change
2000	435,570	—	2,076	—	450,725	—	2,148	—	15,155	—	72	—
2001	426,720	-2.0	2,010	-3.2	440,693	-2.2	2,075	-3.4	13,973	-7.8	66	-8.9
2002	415,724	-2.6	1,936	-3.7	430,763	-2.3	2,006	-3.4	15,040	7.6	70	6.4
2003	400,327	-3.7	1,844	-4.7	415,930	-3.4	1,916	-4.5	15,603	3.8	72	2.6
2004	397,655	-0.7	1,811	-1.8	414,421	-0.4	1,888	-1.5	16,766	7.5	76	6.2
2005	381,098	-4.2	1,717	-5.2	401,187	-3.2	1,807	-4.3	20,089	19.8	90	18.5
2006	380,594	-0.1	1,695	-1.3	401,241	>-0.1	1,787	-1.1	20,648	2.8	92	1.6
2007	361,590	-5.0	1,591	-6.1	384,087	-4.3	1,690	-5.4	22,497	9.0	99	7.7
2008	346,419	-4.2	1,507	-5.3	371,264	-3.3	16,15	-4.5	24,845	10.4	108	9.1
2009	317,736	-8.3	1,367	-9.3	342,124	-7.9	1,472	-8.9	24,388	-1.8	105	-2.9
2010	300,451	-5.4	1,278	-6.5	329,239	-3.8	1,400	-4.9	28,788	18.0	122	16.7
2011	292,769	-2.6	1,232	-3.6	326,577	-0.8	1,374	-1.9	33,808	17.4	142	16.2
% change, from 2000 to 2011	**-32.8**	—	**-40.7**	—	**-27.5**	—	**-36.0**	—	**123.1**	—	**96.9**	

* Adults aged ≥18 years as reported annually by the U.S. Census Bureau.
† Includes cigarettes, small cigars and large cigars, and per-cigarette equivalents for pipe tobacco and roll-your-own tobacco based on the conversion rate in the Master Settlement Agreement: 0.0325 oz (0.9 g) of tobacco = one cigarette.
§ Includes all combustible products other than cigarettes.

TABLE 2. Total consumption of noncigarette combustible tobacco product, by product category and type — United States, 2000–2011

Year	Loose tobacco				Cigars			
	Roll-your-own* (in millions)	% change	Pipe* (in millions)	% change	Small cigars (in millions)	% change	Large cigars (in millions)	% change
2000	5,995	—	2,999	—	2,279	—	3,882	—
2001	4,714	-21.4	2,915	-2.8	2,239	-1.8	4,105	5.7
2002	5,737	21.7	2,757	-5.4	2,343	4.6	4,203	2.4
2003	6,207	8.2	2,389	-13.3	2,474	5.6	4,533	7.9
2004	6,600	6.4	2,314	-3.2	2,917	17.9	4,935	8.9
2005	8,614	30.5	2,423	4.7	3,968	36.0	5,084	3.0
2006	8,594	-0.2	2,322	-4.2	4,434	11.7	5,299	4.2
2007	9,326	8.5	2,463	6.1	5,161	16.4	5,548	4.7
2008	10,721	15.0	2,586	5.0	5,881	14.0	5,657	2.0
2009	6,006	-44.0	6,256	142.0	2,343	-60.2	9,784	73.0
2010	3,168	-47.2	12,351	97.4	983	-58.1	12,287	25.6
2011	2,622	-17.2	17,459	41.4	798	-18.8	12,929	5.2
% change, from 2000 to 2011	**-56.3**	—	**482.1**	—	**-65.0**	—	**233.1**	

* These data are the per-cigarette equivalent based on the conversion rate in the Master Settlement Agreement: 0.0325 oz (0.9 g) of tobacco = one cigarette.

led manufacturers to relabel roll-your-own tobacco as pipe tobacco and then market this relabeled pipe tobacco for roll-your-own use (*7–9*). In addition, manufacturers were able to increase the per-unit weight of certain small cigars to take advantage of a tax benefit when classified as large cigars, which are taxed based on the product price rather than per cigar (*7*). As a result of relatively minor increases in per-unit weight, the new "large cigar" can appear almost identical to a "small cigar," which resembles a typical cigarette and can cost as little as 7 cents per cigar (Figure 1) (*7*).

This analysis shows that cigarette consumption continues to decline in the United States, a trend that has persisted since the 1960s. However, recent changes in consumption patterns, particularly increases in large cigar and pipe tobacco use, have resulted in a slowing of the decline in consumption of all combustible tobacco, and indicate that certain cigarette smokers have switched to using lower-taxed noncigarette combustible products. Moreover, a 2012 Surgeon General's report found that youths and young adults had even higher rates of cigar use and simultaneous use of multiple tobacco products (*10*).

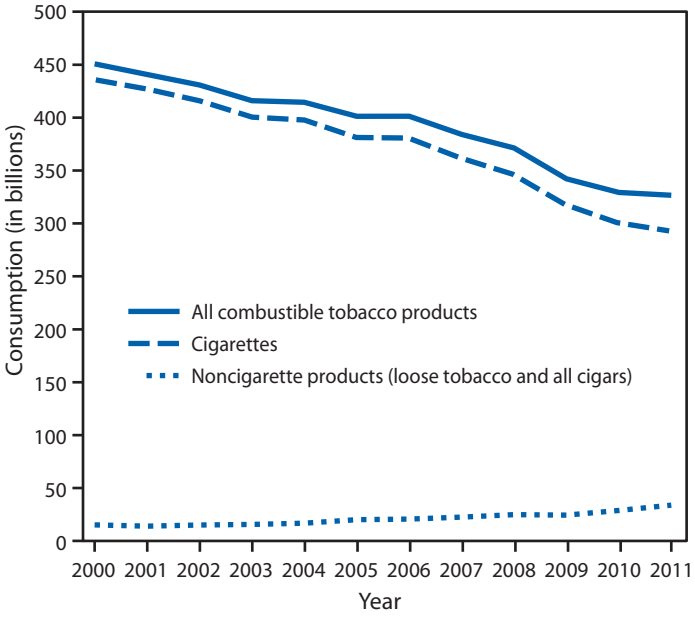
Recent analysis of excise tax data for pipe tobacco, roll-your-own cigarette tobacco, small cigars, and large cigars reveals that the tobacco industry is adapting the marketing and production of cigars and roll-your-own tobacco products to minimize federal excise tax and thus reduce these tobacco products' prices compared with cigarettes (*7–9*). Reducing the effective federal and state excise tax rates on tobacco lessens the impact of cost on reducing smoking and preventing smoking

FIGURE 1. Physical differences between combustible tobacco products — Government Accountability Office, United States

1. Roll-your-own cigarette made by hand with roll-your-own tobacco
2. Roll-your-own cigarette made in a commercial roll-your-own machine with pipe tobacco
3. Factory-made cigarette
4. Small cigar
5. Filtered large cigar
6. Traditional large cigar

Source: Government Accountability Office. Tobacco taxes: large disparities in rates for smoking products trigger significant market shifts to avoid higher taxes. Available at http://www.gao.gov/products/gao-12-475.

FIGURE 2. Consumption of cigarettes and other combustible tobacco products — United States, 2001–2011



initiation. The Government Accountability Office (GAO) recommends modifying federal tobacco taxes to eliminate large tax differentials between roll-your-own and pipe tobacco and small and large cigars (*7*). In addition, because Food and Drug Administration (FDA) regulations currently do not apply to cigars and pipe tobacco, these products can be produced with flavoring, can be labeled with misleading descriptors such as "light" or "low tar," and can be marketed and sold with fewer restrictions than apply to cigarettes.

What is already known on this topic?

Cigarette use continues to decline in the United States, a trend that has persisted since the 1960s.

What is added by this report?

From 2000 to 2011, consumption of all combustible tobacco products decreased from 450.7 billion cigarette equivalents to 326.6 (a 27.5% decrease), and per capita consumption of all combustible tobacco products declined from 2,148 to 1,374 (a 36.0% decrease). However, whereas consumption of cigarettes decreased 32.8%, consumption of noncigarette combustible tobacco increased 123.1%. As a result, the percentage of combustible tobacco consumption composed of loose tobacco and cigars increased from 3.4% in 2000 to 10.4% in 2011.

What are the implications for public health practice?

The increase in cigar and pipe tobacco use is a public health concern because all combustible tobacco use causes cancer, heart disease, and other smoking-related diseases. A switch from cigarettes to other, lower-taxed, combustible tobacco products blunts the effect of increasing prices, one of the most effective ways to reduce smoking and prevent youth smoking initiation.

The findings in this report are subject to at least one limitation. CDC's measure for cigarette and combustible tobacco consumption only accounts for products taxed for legal sale in the United States and does not account for illicit cigarette sales, such as those smuggled into or out of the country, or for untaxed cigarettes that are produced or sold on American Indian sovereign lands. Currently, no method exists for measuring or estimating illicit or untaxed tobacco trade in the United States.

Smoke from pipes and cigars contains the same toxic chemicals as cigarette smoke (*1*). The evidence that the increase in cigar and pipe tobacco use is the result of offering cigarette smokers a low-priced alternative product is a particular public health concern, because the morbidity and mortality effects of other forms of combustible tobacco are similar to those of cigarettes. Increasing prices has been one of the most effective ways to reduce tobacco use and prevent youth smoking initiation (*10*). In addition, combustible tobacco products that are similar in design but not legally considered to be cigarettes are not subject to FDA regulations related to manufacturing, flavoring, labeling, and marketing. The availability of low-priced and less regulated alternative products appears to have led certain cigarette smokers to switch to other combustible tobacco products. This group also might include persons who otherwise might have quit smoking as a result of the 2009 federal tobacco excise tax increase and FDA cigarette regulations. Diminishing the public health impact of excise tax increases and regulation can hamper efforts to prevent youth smoking initiation, reduce consumption, and prompt quitting.

References

1. US Department of Health and Human Services. A report of the Surgeon General: how tobacco smoke causes disease: the biology and behavioral basis for smoking-attributable disease, 2010. Atlanta, GA: US Department of Health and Human Services, CDC; 2010. Available at http://www.surgeongeneral.gov/library/reports/tobaccosmoke/index.html. Accessed July 30, 2012.
2. US Department of Health and Human Services. Ending the tobacco epidemic: a tobacco control strategic action plan for the US Department of Health and Human Services. Washington, DC: Office of the Assistant Secretary for Health; 2010. Available at http://www.hhs.gov/ash/initiatives/tobacco/tobaccostrategicplan2010.pdf. Accessed July 30, 2012.
3. Hatziandreau EJ, Pierce JP, Fiore MC, Grise V, Novotny TE, Davis RM. The reliability of self-reported cigarette consumption in the United States. Am J Public Health 1989;79:1020–3.
4. CDC. Vital Signs: current cigarette smoking among adults aged ≥18 years—United States, 2005–2010. MMWR 2011;60:1207–12.
5. Economic Research Service, US Department of Agriculture. Tobacco outlook. Washington, DC: US Department of Agriculture; 2007. Available at http://usda.mannlib.cornell.edu/usda/current/TBS/TBS-10-24-2007.pdf. Accessed July 30, 2012.
6. Alcohol and Tobacco Tax and Trade Bureau. Tobacco statistics. Washington, DC: US Department of Treasury, Alcohol and Tobacco Tax and Trade Bureau. Available at http://www.ttb.gov/tobacco/tobacco-stats.shtml. Accessed August 1, 2012.
7. Government Accountability Office. Tobacco taxes: large disparities in rates for smoking products trigger significant market shifts to avoid higher taxes. Washington, DC: Government Accountability Office; 2012. Available at http://www.gao.gov/products/gao-12-475. Accessed July 30, 2012.
8. Morris DS. Tobacco manufacturing data demonstrate industry product switching in response to tax increases. Tob Control 2010;19:421–2.
9. Morris DS, Tynan MA. Fiscal and policy implications of selling pipe tobacco for roll-your-own cigarettes in the United States. PLoS One 2012;7:e36487.
10. US Department of Health and Human Services. Preventing tobacco use among youth and young adults, 2012: a report of the Surgeon General. Atlanta, GA: US Department of Health and Human Services, CDC; 2012. Available at http://www.surgeongeneral.gov/library/reports/preventing-youth-tobacco-use/index.html. Accessed July 30, 2012.

Public Health Interventions Involving Travelers with Tuberculosis — U.S. Ports of Entry, 2007–2012

Every day, approximately 950,000 international travelers arrive in the United States (*1*). The Secretary of the U.S. Department of Health and Human Services is authorized to prevent the introduction, transmission, and spread of communicable diseases by travelers into and within the United States (*2*). The Secretary, through the CDC director, delegates this authority to CDC's Division of Global Migration and Quarantine (DGMQ). Of the communicable diseases for which federal quarantine and isolation are authorized by executive orders of the president (*2*), infectious tuberculosis (TB) is encountered most commonly by DGMQ's network of quarantine stations at major U.S. ports of entry (Table). Although legal immigrants and refugees undergo U.S. State Department–mandated TB screening overseas, CDC receives approximately 125 reports each year of arriving travelers with active TB, including foreign visitors, foreign students, and temporary workers (CDC, unpublished data, 2012). This report describes two cases that illustrate the TB control and prevention activities of quarantine stations. Such activities, including issuing federal isolation orders, restricting travel, arranging safe transport for patients across state lines, and conducting airline contact investigations, support CDC's mission to limit the spread of infectious disease from travelers.

Case Reports

Case 1. On March 24, 2010, the Nevada State TB Program notified the CDC Los Angeles Quarantine Station about an elderly legal immigrant from Mexico with infectious TB. The patient was admitted to a Nevada hospital in October 2009. Sputum smears revealed the presence of acid-fast bacilli (AFB), and standard four-drug treatment (isoniazid, rifampin, pyrazinamide, and ethambutol) was started empirically. The local TB clinic provided outpatient treatment under directly observed therapy until December 2009, when the patient abruptly left the United States for Mexico without notifying the clinic, and before drug susceptibility tests showed isoniazid resistance. Local public health officials referred the case to Cure-TB,* a binational TB program that facilitates continuity of care for patients with TB who travel between the United States and Mexico.

The patient returned briefly to the United States in March 2010, but made no contact with local TB control officers and departed again to Mexico. After discussions with state and local public health partners, CDC issued a federal isolation order and placed the patient on public health travel restriction lists (Do Not Board [DNB] and lookout lists) because of the risk for infectiousness resulting from suboptimal treatment, continued nonadherence with public health recommendations, and recent history of international travel. Persons included on the DNB list are assigned a public health lookout record, which alerts Customs and Border Protection (CBP) officers if the person attempts to enter the United States through any port of entry (*3*).

In September 2010, the patient was detected by CBP at a border crossing in El Paso, Texas. The CDC El Paso Quarantine Station served a federal isolation order, and the patient was transported to a nearby Texas hospital under CBP custody for evaluation and treatment. After three sputum specimens tested AFB smear-negative, the patient was escorted by a CDC quarantine public health officer to Nevada. The federal isolation order was rescinded, and the patient was transferred to the custody of a local health department for court-ordered home isolation. Compliance with an effective treatment regimen, administered through directly observed therapy, permitted removal of federal travel restrictions in November 2010.

Case 2. On October 18, 2011, the Ohio Department of Health TB Program reported a college student from China with AFB smear-positive, cavitary TB disease to the CDC Detroit Quarantine Station. In August 2011, the student had traveled from Japan to California on a commercial flight that exceeded 8 hours, and then flew on two connecting domestic flights (California to Illinois and Illinois to Ohio, each of which was <8 hours).

When DGMQ protocol conditions for TB airline contact investigations are met, including infectiousness criteria and flight duration of ≥8 hours, the jurisdictional quarantine station obtains the flight manifest and locator information for potentially exposed passengers on the flight (*4*). State health departments then are notified of contacts in their jurisdictions via the Epidemic Information Exchange (Epi-X), CDC's secure electronic communications network for public health professionals.

The CDC Detroit Quarantine Station obtained the international flight manifest and identified 15 passengers as contacts based on their seat assignments (passengers in the same row, two rows in front of, and two rows behind the index case). DGMQ notified nine state health departments of 11 U.S. resident passenger-contacts and the ministries of health of two countries about four passenger-contacts who lived outside the United States. Outcomes were reported to DGMQ by U.S. health departments for five passenger-contacts. Of those, two

* Additional information available at http://www.sdcounty.ca.gov/hhsa/programs/phs/cure_tb.

TABLE. CDC quarantine stations and the jurisdictions in which they monitor ports of entry, 2012*

Quarantine station	Jurisdiction
Anchorage, Alaska	Alaska
Atlanta, Georgia	Georgia, North Carolina, South Carolina, and Tennessee
Boston, Massachusetts	Massachusetts, Maine, New Hampshire, and Rhode Island
Chicago, Illinois	Illinois, Indiana, Iowa, and Wisconsin; preclearance port in Toronto, Canada
Dallas, Texas	Kansas, Missouri, Oklahoma, Arkansas, and northern Texas (Health districts 1, 2, and 3)
Detroit, Michigan	Michigan, Kentucky, and Ohio
El Paso, Texas (U.S.–Mexico unit)	Western Texas (Health districts 8, 9, 10, and 11) and New Mexico
Honolulu, Hawaii	Hawaii, Guam, and Pacific Trust Territories
Houston, Texas	Eastern Texas (Health districts 4, 5, 6, and 7) and Louisiana
Los Angeles, California	Southern California (Los Angeles, Orange, San Bernardino, Riverside, Ventura, Santa Barbara, San Luis Obispo, Inyo, and Kern counties), Nevada, Utah, and Colorado
Miami, Florida	Florida, Alabama, and Mississippi; preclearance ports in the Bahamas
Minneapolis-St. Paul, Minnesota	Minnesota, Nebraska, North Dakota, and South Dakota
New York, New York	New York, Connecticut, and Vermont; preclearance ports in Montreal, Canada; Bermuda; and Shannon and Dublin, Ireland
Newark, New Jersey	New Jersey
Philadelphia, Pennsylvania	Pennsylvania and Delaware
San Diego, California (U.S.–Mexico unit)	Arizona, California (San Diego and Imperial counties)
San Francisco, California	Central and northern California (46 counties) and Wyoming
San Juan, Puerto Rico	Puerto Rico and the U.S. Virgin Islands
Seattle, Washington	Washington, Idaho, Montana, and Oregon; preclearance ports in Edmonton, Calgary, Vancouver, and Victoria, Canada
Washington, DC	District of Columbia, Maryland, Virginia, and West Virginia

* Additional information available at http://www.cdc.gov/quarantine/quarantinestations.html.

were evaluated and determined not to have been infected with TB; attempts to notify the other three were unsuccessful.

Reported by

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Editorial Note

In 2011, 10,521 new TB cases were reported in the United States, with rates 12 times higher in foreign-born persons than in U.S.-born persons (*5*). From June 2007 to December 2011, 632 cases of active TB among travelers were reported to CDC quarantine stations (CDC, unpublished data, 2012). TB transmission during air travel has been documented (*4,6*), but the risk for transmission has not been determined and is believed to be low. One model estimates the risk for transmission from a highly infectious passenger on an 8.7-hour commercial flight as 1 per 1,000 for all passengers, with higher risk to those seated closer to the infectious passenger (*7*). Delegated authority permits DGMQ's use of public health travel restriction tools and federal isolation orders to prevent persons known or suspected of having infectious TB from traveling. These tools can facilitate the safe transport of travelers with TB to local hospitals or their home states for testing and continued treatment. Since June 2007, five federal isolation orders have been served to persons with TB (inclusive of case 1), four of whom were foreign-born; before 2007, the last federal isolation order was issued in 1963.

Domestic or international public health officials may request that a person be placed on the DNB and lookout lists, which have been managed jointly by CDC and the Department of Homeland Security since formalization of the process in June 2007 (*3*). If persons on the lists are identified at ports of entry, CBP notifies the jurisdictional quarantine station to facilitate public health clearance or action. From June 2007 to December 2011, 205 persons with known or suspected TB were added to the DNB and lookout lists; 173 (84%) have since been removed after meeting criteria indicating noninfectiousness (CDC, unpublished data, 2012). The first case report, involving multiple health jurisdictions and CDC quarantine stations, exemplifies the successful use of the lookout record to intercept a TB-infected traveler at a land border and return the patient to public health management. The federal isolation order had been drafted months before the patient was encountered at the port of entry, facilitating immediate medical evaluation and return of the patient to health care in his home state.

The second case report highlights CDC quarantine stations' response to notifications of travelers with infectious TB who traveled by commercial aircraft. From June 2007 to December 2011, CDC quarantine stations, in collaboration with U.S. health departments, performed airline contact investigations for 390 travelers with infectious TB, involving 508 flights with approximately 15,650 potentially exposed contacts. DGMQ also notified foreign public health authorities in more than 50 countries of at least 3,000 international contacts

What is already known on this topic?

The global burden of tuberculosis (TB) and the tremendous volume of travelers to the United States increase the risk for TB importation and transmission during travel. Significant resources are expended during public health responses to travelers with TB disease, including passenger contact investigations, legal measures, and implementation of federal travel restriction tools.

What is added by this report?

The case studies in this report illustrate the use of federal legal measures and travel restriction tools to help return noncompliant TB-infected persons to public health care, and highlight revised guidelines to optimize the cost-benefit ratio of airline TB contact investigations.

What are the implications for public health practice?

TB control in travelers into and within the United States can be promoted through ongoing state and local public health practitioner partnerships with their jurisdictional CDC quarantine stations and referral of immigrants with noninfectious TB conditions at ports of entry to TB clinics in their destination states.

(CDC, unpublished data, 2012). However, because outcome reporting to CDC is voluntary, contact tracing outcome reports typically are received for <20% of passenger contacts (*4*). In 2011, DGMQ used the results of epidemiologic and economic impact evaluations to revise its criteria for conducting airline contact investigations (Box). The policy changes conserve state and federal public health resources by assigning priority for tracing to the passenger-contacts of travelers who are most likely to transmit *Mycobacterium tuberculosis* (those with both positive sputum AFB smears and cavitation identified on chest radiograph) or who have multidrug-resistant TB. CDC quarantine stations also provide guidance to crews on ships regarding TB contact investigations when notified of travelers with infectious TB on maritime vessels.

In addition to responding to reports of infectious TB in travelers, four CDC quarantine stations meet immigrants arriving at U.S. ports of entry who have been diagnosed with admissible, noninfectious TB conditions during their pre-immigration medical screening, and provide them with a TB clinic referral in the states of their destination. Immigrants receiving referrals are four times more likely to initiate follow-up evaluation than those receiving no referral (p<0.001; CDC, unpublished data, 2012). Immigrants typically are not charged for these medical evaluations; the costs usually are borne by state and local health departments. Follow-up is important because newly arrived U.S. immigrants with a history of TB infection or previously treated disease have an increased risk for disease activation or reactivation during their first few years after arrival (*8*). DGMQ is developing a system to expand the referral program to include more CDC quarantine stations.

BOX. CDC criteria for initiating flight-related tuberculosis contact investigations, June 2011

- Index case was diagnosed within 3 months of the flight AND the flight occurred within 3 months of notification to the Division of Global Migration and Quarantine.
- Flight lasted ≥8 hours gate-to-gate.*
- Diagnosis of the index case was confirmed by sputum culture or nucleic acid amplification test for *Mycobacterium tuberculosis* AND is:
  1. Sputum smear-positive for acid-fast bacilli AND cavitation is present on a chest radiograph; OR
  2. Confirmed to have a multidrug-resistant isolate (regardless of the smear or chest radiograph results).

Note: A contact investigation will be considered on a case-by-case basis for situations that are unusual or not clearly addressed by the criteria. Examples include, but are not limited to, situations in which an unusually high proportion of close contacts have positive tuberculin skin test or interferon-gamma release assay test screening results, an index case has laryngeal tuberculosis, or cavitation is detected on chest computed tomography scan but no chest radiograph was performed.

* Gate-to-gate means all time spent on the aircraft, including boarding and deplaning time or delays on the tarmac.

The network of CDC quarantine stations provides national leadership and coordination of public health responses to TB in travelers. DGMQ also communicates with foreign health authorities about TB patients or contacts who are no longer in the United States, and collaborates with U.S. health departments to work with TB patients who have left the United States but could return. Effective collaboration between CDC quarantine stations and international, state, and local public health practitioners can help reduce the spread of TB during travel by intercepting TB patients at ports of entry, returning patients to treatment, and identifying contacts for possible intervention.

Acknowledgments

Applied Epidemiology Fellowship Program, Council of State and Territorial Epidemiologists. State and local health departments. Quarantine stations, Joaquin Rueda, Chris Schembri, MPH, Rebecca Wong, MPH, Div of Global Migration and Quarantine, CDC.

References

1. US Customs and Border Protection. On a typical day in fiscal year 2010, CBP... Washington, DC: US Customs and Border Protection, Department of Homeland Security; 2011. Available at http://www.cbp.gov/xp/cgov/about/accomplish/previous_year/fy10_stats/typical_day_fy2010.xml. Accessed March 25, 2012.
2. CDC. Legal authorities for isolation and quarantine. Atlanta, GA: US Department of Health and Human Services, CDC; 2012. Available at http://www.cdc.gov/quarantine/aboutlawsregulationsquarantineisolation.html. Accessed February 3, 2012.
3. CDC. Federal air travel restrictions for public health purposes—United States, June 2007–May 2008. MMWR 2008;57:1009–12.
4. Marienau KJ, Burgess GW, Cramer E, et al. Tuberculosis investigations associated with air travel: U.S. Centers for Disease Control and Prevention, January 2007–June 2008. Travel Med Infect Dis 2010;8:104–12.
5. CDC. Trends in tuberculosis—United States, 2011. MMWR 2012; 61:181–5.
6. Abubakar I. Tuberculosis and air travel: a systematic review and analysis of policy. Lancet Infect Dis 2010;10:176–83.
7. Ko G, Thompson KM, Nardell EA. Estimation of tuberculosis risk on a commercial airliner. Risk Anal 2004;24:379–88.
8. McKenna MT, McCray E, Onorato I. The epidemiology of tuberculosis among foreign-born persons in the United States, 1986–1993. N Engl J Med 1995;332:1071–6.

Infant Lead Poisoning Associated with Use of Tiro, an Eye Cosmetic from Nigeria — Boston, Massachusetts, 2011

Lead is highly toxic and can damage the brain, kidneys, bone marrow, and other body systems; high levels can cause convulsions, coma, and death (*1*). Young children are especially susceptible to lead exposures because of their floor-hand-mouth activity, greater gut absorption, and developing central nervous systems. In June 2011, a male infant aged 6 months of Nigerian descent was referred to the Pediatric Environmental Health Specialty Unit (PEHSU) at Boston Children's Hospital because of an elevated blood lead level (BLL). An investigation found no lead exposure except for "tiro," a Nigerian cosmetic that also is used as a folk remedy to promote visual development. The tiro applied to the infant's eyelids contained 82.6% lead. Products similar to tiro, such as "surma" and "kajal" in Asia and kohl in the Middle East, also might contain lead. This case adds to the medical literature documenting nonpaint lead sources as causes of elevated BLLs in children (*2,3*) and highlights persons of certain immigrant populations as a risk group. Educational efforts are needed to inform immigrants from Africa, Asia, and the Middle East that tiro and similar products can cause lead poisoning in children. Health-care providers and public health workers should ask about eye cosmetics and folk remedies when seeking a source of exposure in children with elevated BLLs from certain immigrant populations.

In June 2011, during a well-child visit of a male infant aged 6 months born in the United States to Nigerian parents, the physician noted that an imported cosmetic had been applied to the child's eyelids. Capillary blood testing performed by the physician indicated a BLL of 13 μg/dL, more than twice the CDC's reference value of 5 μg/dL, based on the 97.5th percentile of the BLL distribution in U.S. children aged 1–5 years. The next day, a confirmatory venous BLL measured by graphite furnace atomic absorption spectroscopy was 12 μg/dL. Additional laboratory evaluation revealed a normal hemoglobin level and 2+ erythrocyte microcytosis on an automated blood smear. In accordance with CDC recommendations aimed to help reduce the absorption of lead and mitigate the severe adverse health effects of lead exposure,* the pediatrician prescribed supplemental iron, contacted the Massachusetts Department of Public Health, and referred the family to the regional PEHSU.

When the infant was brought to the PEHSU 1 week later, his venous BLL, as measured by the same laboratory, was 13 μg/dL. His whole blood zinc protoporphyrin (30 μg/dL whole blood [normal: 0–35 μg/dL]), hemoglobin (12.1 g/dL [normal: 10.4–12.5 g/dL]), erythrocyte mean cell volume (74.2 fL [normal: 68.0–83.1 fL]), plasma iron (81 μg/dL [normal: 40–100 μg/dL]), and ferritin (65.0 ng/mL [normal: 10.0–75.0 ng/mL]) were in the normal range for his age. A manual blood smear showed 2+ erythrocyte microcytosis. The parents reported no health concerns for the infant, and a detailed review of systems was normal. The infant had no relevant past medical history, was growing well, and had met all developmental milestones. No other children lived in the home. Both parents had sickle cell trait; the infant had a normal hemoglobin electrophoresis. No abnormalities were noted on the physical examination.

Since 2008, the family had lived in a townhouse originally built in 2004. PEHSU staff members inspected the residence and found it to be in excellent condition, without lead hazards. Other sources of lead exposure were ruled out, including take-home exposure from parental occupations, kitchenware, family hobbies, and diet. The infant was breastfed exclusively and did not consume any imported herbs, spices, or dietary supplements. Additional questioning revealed that since age 2 weeks, a Nigerian cosmetic and folk remedy had been applied to the infant's eyelids three to four times weekly to improve attractiveness and promote visual development. A grandparent had purchased the powder, called tiro (Figure 1), from a street vendor in Ilorin, a city in Kwara State, Nigeria. The PEHSU recommended immediately discontinuing the use of tiro on the infant and continuing iron supplementation. The parents agreed to submit the suspected tiro powder for laboratory analysis.

Quantitative analysis by the PEHSU showed that the tiro consisted of 82.6% lead. A single application of 10 mg of tiro would deliver 8 mg of lead to the infant's eyelids. The most likely routes of exposure were eyelid-hand-mouth and absorption from the conjunctival surfaces of the eyes or in ingested tears. Analysis of the tiro by the U.S. Geological Survey, using scanning electron microscopy (SEM), showed that the sample was dominated by lead sulfide, known as galena (Figure 2), which has relatively low bioavailability (*1*). No other minerals were observed by SEM, although small amounts of other minerals commonly found as microscopic inclusions in lead sulfide might have escaped detection.

Three months after the family stopped applying tiro to the infant's eyelids, his venous BLL had fallen from 13 μg/dL to 8 μg/dL.

* Recommendations available at http://www.cdc.gov/nceh/lead/casemanagement/casemanage_chap4.htm.

FIGURE 1. The Nigerian tiro container and the powder that was applied to the lead-poisoned child's eyelids — Boston, Massachusetts, 2011

Photo/Pediatric Environmental Health Specialty Unit, Boston Children's Hospital

Reported by

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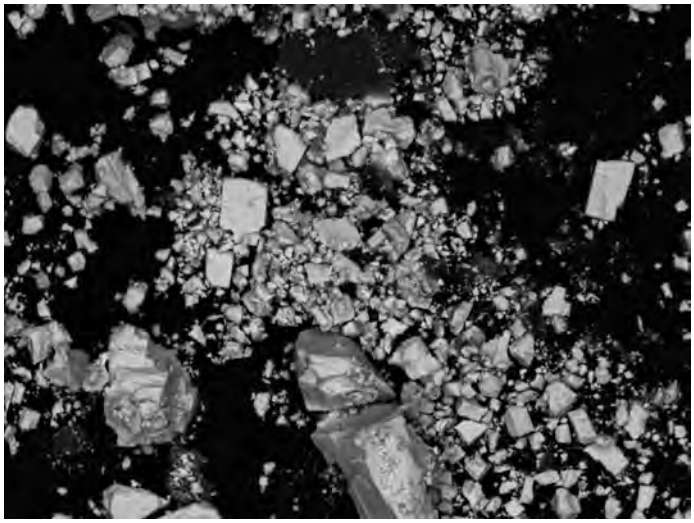
Editorial Note

Although the primary source of lead exposure in the United States is lead-based paint, nonpaint sources of lead increasingly are being identified in lead poisoning cases (*2,3*). These nonpaint exposures include recent travel to a foreign country, take-home exposure when persons exposed to lead at their workplace contaminate their homes or vehicles, and use of imported products such as spices, food, candy, cosmetics, health remedies, ceramics or pottery, and jewelry.

This report describes an eye cosmetic and folk remedy as the source of lead poisoning in a child of Nigerian descent; a similar case has been reported in the United Kingdom (*4,5*). Although Nigeria switched to unleaded gasoline by the end of 2003, Nigerian children might also be exposed to the lead that remains in the soil from years of use of leaded gasoline. In addition, lead contamination resulting from gold mining has caused many child deaths in Nigerian villages where artisanal gold ore processing takes place (*6,7*).

Tiro is the Yoruba name for this eye cosmetic implicated in the case described in this report. In another Nigerian language, Hausa, it is called "tozali" or "kwalli." Similar products intended to darken the eyes are known as kohl in English and Arabic and as "surma" or "kajal" in languages spoken in India and Pakistan. These preparations are not standardized, and not all contain lead. One alternative to lead sulfide is another toxic compound, antimony sulfide. Imported cosmetics are one of the relatively few sources of significant lead exposure for infants too young to crawl or walk; however, exposure to lead in tiro represents an additional burden to groups who might be exposed to other sources of lead. The contribution that tiro might make to the cumulative burden of lead poisoning should not be overlooked.

FIGURE 2. Scanning electron microscopy* of the tiro eye cosmetic powder that was applied to the lead-poisoned child's eyelids, revealing the presence of cubic shapes and stair-step cleavage, both of which indicate presence of lead sulfide (also known as galena) — Boston, Massachusetts, 2011



Photo/U.S. Geological Survey, Crustal Geophysics and Geochemistry Science Center
* Field of view is approximately 100 μm wide.

This fine powder is applied to the dermal surfaces of the eyelid. In addition to its use by the patient's family for improving attractiveness and promoting visual development, tiro has been used to ward off "the evil eye"; to relieve eyestrain, pain, or soreness; to prevent infection of the umbilical stump or a circumcision wound by local application; and to prevent sun glare (*8,9*).

This case identifies tiro as a potential lead exposure among not only Nigerians living in the United States, but also among African, Asian, and Middle Eastern populations who use similar products. Public health educational campaigns can help identify and prevent further cases (*10*).† Obstetricians, pediatricians, midwives, and allied health-care professionals

† Examples of such campaigns are described at http://www.nyc.gov/html/doh/html/lead/lead-import-eyecos.shtml.

should discuss this potential risk factor during prenatal and early childhood medical visits by families for whom these cultural practices might apply. Although CDC recommends blood lead testing for internationally adopted and refugee children,[§] blood lead testing in children of certain immigrant populations also might be important because of the increased risk for exposure to lead-containing foreign products.

The Nigeria Centre for Disease Control is working with the vendors of products such as tiro to find possible safer alternatives. Discussions involve the perceived benefit of tiro, and evidently, strong beliefs are attached to its use. The Nigeria Centre for Disease Control plans to launch a national public health awareness campaign.

[§] Guidelines available at http://www.cdc.gov/nceh/lead/tips/populations.htm.

Acknowledgment

Geoffrey S. Plumlee, PhD, US Geological Survey, Crustal Geophysics and Geochemistry Science Center, Denver, Colorado.

References

1. Agency for Toxic Substances and Disease Registry. Toxicological profile for lead. Atlanta, GA: US Department of Health and Human Services, CDC, Agency for Toxic Substances and Disease Registry; 2007. Available at http://www.atsdr.cdc.gov/toxprofiles/tp13.pdf. Accessed July 26, 2012.
2. Gorospe EC, Gerstenberger SL. Atypical sources of childhood lead poisoning in the United States: a systematic review from 1966–2006. Clin Toxicol (Phila) 2008;46:728–3.
3. CDC. Childhood lead poisoning associated with tamarind candy and folk remedies—California, 1999–2000. MMWR 2002;51:684–6.
4. Warley MA, Blackledge P, O'Gorman P. Lead poisoning from eye cosmetic. Brit Med J 1968;1:117.
5. Healy MA, Aslam M, Bamgboye OA. Traditional medicine and lead-containing preparations in Nigeria. Public Health 1984;98:26–32.
6. CDC. Notes from the field: Outbreak of acute lead poisoning among children aged <5 years—Zamfara, Nigeria, 2010. MMWR 2010;59:846.
7. Dooyema CA, Neri A, Lo YC, et al. Outbreak of fatal childhood lead poisoning related to artisanal gold mining in northwestern Nigeria, 2010. Environ Health Perspect 2012;120:601–7.
8. Al-Hawi SA, Wafai MZ, Kalaagi MR, Al-Ugum WA. Light of the eyes and the collector of arts, vol. 1286. Riyadh, Saudi Arabia: King Faisal Center for Research and Islamic Studies; 1980:142.
9. Hardy AD, Vaishnav R, Al-Kharusi SSZ, Sutherland HH, Worthing MA. Composition of eye cosmetics (kohls) used in Oman. J Ethnopharmacol 1998;60:223–34.
10. Woolf AD, Hussain J, McCullough L, Petranovic M, Chomchai C. Infantile lead poisoning from an Asian tongue powder: a case report & subsequent public health inquiry. Clin Toxicol (Phila) 2008;46:841–4.

What is already known on this topic?

Although the most common source of lead poisoning for young children in the United States is lead-based paint, nonpaint sources of lead are being identified increasingly in lead poisoning cases, particularly in immigrant communities.

What is added by this report?

A male infant aged 6 months was found to have an elevated blood lead level (BLL) attributed to application of "tiro," a Nigerian eye cosmetic, to his eyes by his parents. Tiro, also known as "tozali" and "kwalli" in Nigeria, is similar to kohl, "surma," and "kajal" used in the Middle East, India, and Pakistan. These products often are made with lead. In this case, the lead content was 82.6%. This case adds to the medical literature documenting nonpaint lead sources as causes of elevated BLLs in children and highlights persons of certain immigrant populations as a risk group.

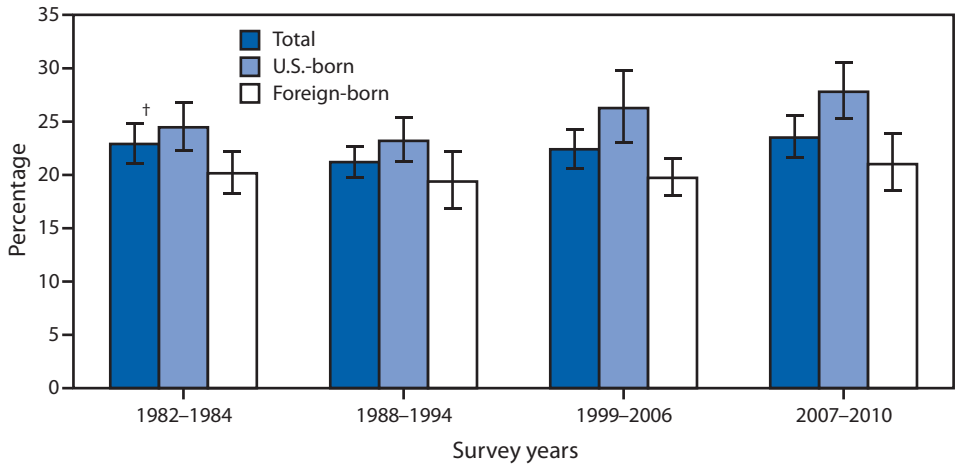
What are the implications for public health practice?

Educational and other primary prevention efforts are needed to inform immigrants from Africa, Asia, and the Middle East that tiro and similar products can cause lead poisoning in children. Health-care providers and public health workers should ask about eye cosmetics and folk remedies when seeking a source of exposure in children with elevated BLLs from certain immigrant populations.

QuickStats

FROM THE NATIONAL CENTER FOR HEALTH STATISTICS

Prevalence of Hypertension Among Mexican-American Adults Aged 20–74 Years, by Country of Birth — United States, 1982–1984 to 2007–2010*



* Age-adjusted to year 2000 U.S. Census Bureau estimates using age groups 20–39 years, 40–59 years, and 60–74 years. Hypertension is defined as a systolic blood pressure ≥140 mmHg, a diastolic blood pressure ≥90 mmHg, or currently taking medication to lower high blood pressure.
† 95% confidence interval.

Mexican-American adults who were born in the United States were more likely to have hypertension compared with those born outside of the United States. From 1982–1984 to 2007–2010, a statistically significant increase in hypertension (from 24.5% to 27.8%) was observed only among those who were born in the United States.

Sources: Fryar CD, Wright JD, Eberhardt MS, Dye BA. Trends in nutrient intakes and chronic health conditions among Mexican-American adults, a 25-year profile: United States, 1982–2006. Natl Health Stat Rep 2012(50).

CDC. Hispanic Health and Nutrition Examination Survey, data for 1982–1984.

CDC. National Health and Nutrition Examination Survey, data for 1988–1994, 1999–2006, and 2007–2010.

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The *Morbidity and Mortality Weekly Report (MMWR)* Series is prepared by the Centers for Disease Control and Prevention (CDC) and is available free of charge in electronic format. To receive an electronic copy each week, visit *MMWR*'s free subscription page at *http://www.cdc.gov/mmwr/mmwrsubscribe.html*. Paper copy subscriptions are available through the Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402; telephone 202-512-1800.

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U.S. Government Printing Office: 2012-523-043/02023 Region IV ISSN: 0149-2195

Int. J. Environ. Res. Public Health **2014**, *11*, 10345-10361; doi:10.3390/ijerph111010345

OPEN ACCESS

International Journal of
Environmental Research and Public Health
ISSN 1660-4601
www.mdpi.com/journal/ijerph

Article

Reasons for Starting and Stopping Electronic Cigarette Use

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External Editor: Konstantinos Farsalinos

Received: 29 June 2014; in revised form: 28 September 2014 / Accepted: 28 September 2014 / Published: 3 October 2014

Abstract: The aim of our study was to explore reasons for starting and then stopping electronic cigarette (e-cigarette) use. Among a national sample of 3878 U.S. adults who reported ever trying e-cigarettes, the most common reasons for trying were curiosity (53%); because a friend or family member used, gave, or offered e-cigarettes (34%); and quitting or reducing smoking (30%). Nearly two-thirds (65%) of people who started using e-cigarettes later stopped using them. Discontinuation was more common among those whose main reason for trying was not goal-oriented (e.g., curiosity) than goal-oriented (e.g., quitting smoking) (81% *vs.* 45%, $p < 0.001$). The most common reasons for stopping e-cigarette use were that respondents were just experimenting (49%), using e-cigarettes did not feel like smoking cigarettes (15%), and users did not like the taste (14%). Our results suggest there are two categories of e-cigarette users: those who try for goal-oriented reasons and typically continue using and those who try for non-goal-oriented reasons and then typically stop using. Research should distinguish e-cigarette experimenters from motivated users whose decisions to discontinue relate to the utility or experience of use. Depending on whether e-cigarettes prove to be effective smoking cessation tools or

whether they deter cessation, public health programs may need distinct strategies to reach and influence different types of users.

Keywords: electronic cigarettes; e-cigarettes; tobacco use; smoking cessation

1. Introduction

The popularity of electronic cigarettes (e-cigarettes) in the United States has increased dramatically in recent years. Less than 1% of U.S. adults reported ever trying e-cigarettes in 2009 [1]; in 2013, 15% reported ever trying them [2]. Use of e-cigarettes by smokers is particularly high. In a 2013 national U.S. survey, half of smokers reported ever trying e-cigarettes, and 21% reported currently using them some days or every day [3]. Use by smokers is similarly high in other countries [4,5]. For instance, more than one-third (37%) of current smokers and recent ex-smokers in Great Britain reported ever use of e-cigarettes in 2012, and 21% currently used them [6].

Among the most common reasons for using e-cigarettes are believing they are healthier than regular cigarettes and can help smokers quit or reduce smoking [7]. Because e-cigarettes do not rely on combustion or contain cured tobacco, they produce far fewer harmful constituents than regular cigarettes [8]. However, some studies of e-cigarette aerosol and e-liquid have detected formaldehyde, nitrosamines, and other harmful constituents in amounts that varied considerably across brands, flavors, and models [9–11]. The extent to which e-cigarettes help smokers quit is still unclear [12–14]. One randomized-controlled trial found similar cessation success rates for e-cigarettes and the nicotine patch [15]. Some population-based studies indicate that e-cigarettes can help smokers quit [16], while others do not [17,18]. Other reported reasons for trying or using e-cigarettes are avoiding smoking restrictions [19–21], alleviating cravings for nicotine [20,22], enjoying the flavors [23], and preventing others' exposure to cigarette smoke [21].

There is little research on stopping use of e-cigarettes outside of surveys with purposeful sampling of e-cigarette users from online forums [20,22] or smokers exiting tobacco shops [24]. It is important to understand why people stop using e-cigarettes for multiple reasons. First, e-cigarettes may provide an opportunity for harm reduction should nicotine-addicted smokers be willing to switch from regular cigarettes to non-combustible electronic ones. Only if switching is both successful and widespread would their use offer a net public health benefit. Second, understanding who stops using e-cigarettes and why allows researchers to distinguish among user types. Just as there are subtypes of cigarette smokers, like "social smokers" [25], subtypes of e-cigarette users may also exist. Reaching the right users with the right messages is critical for the success of any future public health campaigns focused on e-cigarettes. The aim of the present study was to investigate reasons for starting and stopping use of e-cigarettes and to examine differences in discontinuation by reason for trying among a large, population-based sample of U.S. adults.

2. Methods

2.1. Sample

This survey relied on data collected as part of the Tobacco Control in a Rapidly Changing Media Environment (TCME) study. The TCME project examined the relationship between recall of receiving tobacco-related information through multiple media channels and tobacco-related attitudes, beliefs, and behavior. In March 2013, 17,522 U.S. adult TCME participants completed an online survey. Most respondents (75%) were members of KnowledgePanel, a nationally representative online survey panel constructed using random-digit dialing, supplemented by address-based sampling to capture cell phone-only households. The remainder of the sample constituted members of a separate consumer survey panel recruited through online ads for joining the panel. The survey company screened individuals who were part of this consumer sample based on demographics and tobacco use behaviors and issued invitations to this survey in order to quota match with the KnowledgePanel sample. KnowledgePanel members received monthly compensation for being part of the panel (usually as points redeemable for $4–6 of goods) as well as entry into a sweepstakes with an additional cash or prize reward for completing this specific survey. Respondents from the separate consumer panel received points worth up to $2 for taking this survey. Before taking the survey, all participants provided consent online. Of the 34,097 KnowledgePanel members surveyed, 61% completed the screening; among those who were eligible (n = 13,531), 97% completed the survey. Response rates for the convenience sample cannot be calculated because there is no known sampling frame. For this study, we report data only from the 3878 participants who reported ever having tried an e-cigarette. Institutional review boards at the University of Illinois and the National Cancer Institute approved the study.

2.2. Measures

While viewing generic images of e-cigarettes, participants read an introductory statement: “The next questions are about electronic cigarettes, often called e-cigarettes. An e-cigarette looks like a regular cigarette, but it runs on a battery and produces vapor instead of smoke. There are many types of e-cigarettes. Some common brands are Smoking Everywhere, NJOY, Blu, and Vapor King. Below are some pictures of e-cigarettes.” Participants who were aware of e-cigarettes (assessed with the item, “Before today, had you ever heard of e-cigarettes?”) answered items about ever use (“Have you ever used an e-cigarette, even one puff?”) and current use (“Do you now use e-cigarettes every day, some days, or not at all?”). We defined “trying” or “starting” as ever use and “current use” as using e-cigarettes either every day or some days. We defined “stopping” or “discontinuing” as ever use without current use.

One survey item assessed reasons for trying e-cigarettes: “What are the reasons you first tried e-cigarettes? (check all that apply).” The response options were: they are affordable; I can use them in places where smoking cigarettes isn’t allowed; they might be less harmful to me than regular cigarettes; they might be less harmful to people around me than regular cigarettes; e-cigarettes come in flavors I like; e-cigarettes can help me quit or cut back on smoking regular cigarettes; e-cigarettes don’t smell bad; using an e-cigarette feels like smoking a regular cigarette; e-cigarettes don’t bother

people who don't use tobacco; the advertising for e-cigarettes appeals to me; they help me deal with cravings to smoke; I have a friend or family member who uses e-cigarettes; I was curious about e-cigarettes; I received an e-cigarette as a holiday gift; I made a New Year's resolution to quit smoking regular cigarettes; and other (please specify). We recoded open-ended responses that clearly fit into one of the available response options. For example, we recoded a response of "So I can quit smoking" into the category "e-cigarettes can help me quit or cut back on smoking regular cigarettes".

Based on the responses to the open-ended item, we created an omnibus category for the reason "a friend or family member used, gave, or offered e-cigarettes"; this category included positive responses for "I have a friend or family member who uses e-cigarettes", "I received an e-cigarette as a holiday gift", and any open-ended responses mentioning a gift or offer from a friend or family member. We also asked respondents to select the main reason they tried e-cigarettes out of all of the reasons they selected on the previous item. We categorized the main reason as "non-goal-oriented" if respondents said they tried e-cigarettes because of curiosity, a friend or family member, liking the ads, other (non-recoded responses), or said they did not know. We classified the remaining main reasons (e.g., stopping or reducing smoking, using where smoking is not allowed) as "goal-oriented".

Another survey item assessed reasons for stopping e-cigarette use, among those who reported no longer using them: "What are the reasons you stopped using e-cigarettes? (check all that apply)." Response options were: e-cigarettes cost too much money; I didn't like how they tasted; using e-cigarettes didn't help me deal with cravings to smoke; using e-cigarettes didn't help me quit or cut back on smoking regular cigarettes; using e-cigarettes didn't feel like smoking regular cigarettes; e-cigarettes are poor quality, defective, or break easily; I was concerned about the health risks caused by using e-cigarettes; I didn't like the side effects of using them; I was just experimenting with e-cigarettes; and other (please specify). Again, we recoded open-ended responses that clearly fit into one of the available response options.

The survey also assessed use of regular cigarettes, gender, age, education, race and ethnicity, marital status, employment, region of residence, and household income. We classified "non-smokers" as those who had smoked fewer than 100 cigarettes in their lifetimes and "former smokers" as those who smoked 100 or more cigarettes but did not currently smoke. Current daily and non-daily smokers received a question about their intentions to quit ("Do you plan to quit smoking for good…?" with six response options: in the next 7 days, in the next 30 days, in the next 6 months, in the next year, more than one year from now, or I do not plan to quit smoking for good). To assess understanding and appropriateness of item wording and ease of responding to survey items, we conducted cognitive interviews and refined the list of reasons for starting and stopping use of e-cigarettes based on this feedback. We pre-tested the revised survey with 160 respondents. For all variables, we recoded missing scores (<0.5% for each item) to the mean or mode of that item.

2.3. Data Analysis

We examined bivariate associations between respondent characteristics and the three most common reasons for trying e-cigarettes using logistic regression. We included all statistically significant bivariate correlates ($p < 0.05$) in a multivariate model. We used the same procedure to examine associations between respondent characteristics and stopping use of e-cigarettes. To compare rates of

discontinuation among those who tried e-cigarettes for goal-oriented *vs.* non-goal-oriented reasons, we conducted an additional logistic regression with discontinuation as the dependent variable and reason type (goal-oriented *vs*. non-goal-oriented) as the independent variable. Analyses were run in Stata Version 12. Frequencies are unweighted. Percentages and all other analyses used the "svy" command and post-stratification weights to adjust for the representativeness of the sample compared to the U.S. population and the sampling design, including the combination of probability and non-probability samples. Statistical tests were two-tailed with a critical alpha of 0.05.

3. Results

3.1. Participant Characteristics

Most participants (*n* = 3878 ever users of e-cigarettes) were non-Hispanic White (71%), married or living with a partner (57%), and under age 45 (56%) (Table 1). About half were female (51%) and had at least some college education (51%). Most participants were current daily or non-daily smokers (61% and 11%, respectively) or former smokers (19%). Most smokers intended to quit either in the next year (26%) or more than one year from now (58%), but 16% did not intend to quit.

Table 1. Sample characteristics (*n* = 3878 e-cigarette ever users).

Characteristic	*n*	Weighted%
Participant		
Gender		
Male	1544	49.2%
Female	2334	50.8%
Age		
18–24	520	14.6%
25–34	878	27.5%
35–44	585	14.0%
45–54	789	23.5%
55–64	712	13.5%
65 or older	394	6.9%
Education		
Less than high school	213	8.9%
High school	995	39.9%
Some college	1837	36.8%
Bachelor's degree or higher	833	14.3%
Smoking status		
Non-smoker [a]	158	9.7%
Former smoker [b]	392	18.7%
Current non-daily smoker	466	10.7%
Current daily smoker	2862	60.9%
Intention to quit smoking [c]		
In the next year	850	26.3%
More than 1 year from now	2003	58.3%
Do not plan to quit	475	15.5%

Table 1. *Cont.*

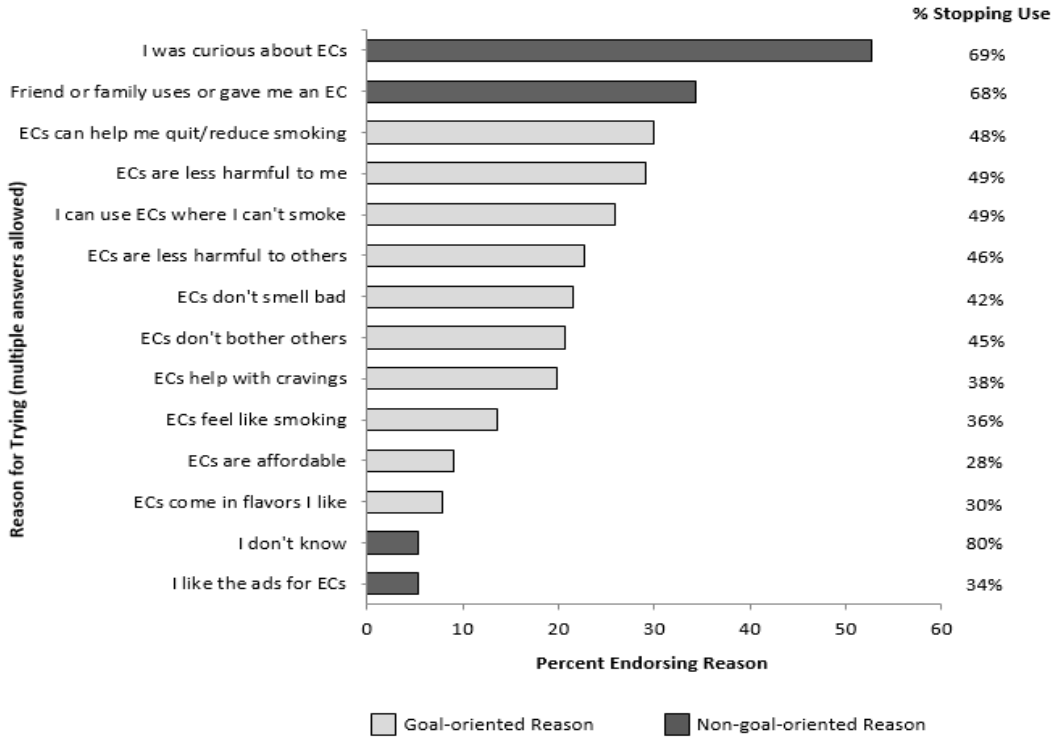
Characteristic	*n*	Weighted%
E-cigarette use		
Former user	2281	65.2%
Current user [d]	1597	34.8%
Race/ethnicity		
Non-Hispanic White	3072	71.0%
Non-Hispanic Black	248	9.2%
Non-Hispanic other race or >1 race	259	6.7%
Hispanic	299	13.2%
Marital status		
Married or living with partner	2169	57.1%
Widowed	145	3.0%
Divorced or separated	714	16.3%
Never married	850	23.6%
Employment		
Working	2150	59.2%
Not working: laid off or looking for work	517	14.2%
Not working: retired, disabled, or other	1211	26.6%
Household		
Region		
Midwest	955	23.5%
Northeast	608	14.6%
South	1360	39.7%
West	955	22.2%
Household income		
Less than $25,000	1130	26.1%
$25,000–$49,999	1153	25.0%
$50,000–$74,999	793	19.6%
$75,000–$99,999	415	16.0%
$100,000 or more	387	13.2%

Notes: [a] Smoked less than 100 cigarettes in lifetime; [b] Smoked 100 or more cigarettes in lifetime but does not currently smoke; [c] Among current smokers (n = 3328); [d] Uses e-cigarettes some days or every day.

3.2. Starting E-Cigarette Use

The most common reasons for starting e-cigarette use were curiosity (53%), a friend or family member used, gave, or offered an e-cigarette (34%), and to quit or cut back on smoking (30%) (Figure 1). Other common reasons for trying e-cigarettes included believing that e-cigarettes were less harmful to the user than regular cigarettes (29%), could be used in places where smoking is not allowed (26%), or were less harmful to others (23%). In open-ended responses, 21 participants (0.4%) noted that they started using e-cigarettes because of price promotions or free samples.

Figure 1. Reasons for trying e-cigarettes (ECs) (multiple answers allowed) and percent stopping EC use among those who endorsed that reason (*n* = 3878).



In multivariate regression analyses, few demographic variables predicted the most common reasons for trying e-cigarettes. Those with some college education were more likely than those with less than a high school education to try e-cigarettes because of curiosity (57.2% *vs*. 40.7%, OR 1.83, 95% CI 1.20, 2.81). Women were more likely than men to report trying e-cigarettes because a friend or family member used, gave, or offered them (38.4% *vs*. 30.1%, OR 1.45, 95% CI 1.16, 1.82). Hispanic participants were less likely than non-Hispanic White participants to try e-cigarettes in order to quit or cut back on smoking (14.9% *vs*. 33.7%, OR 0.50, 95% CI 0.33, 0.76). Former smokers (24.2%, OR 7.68, 95% CI 2.05, 28.72), current non-daily smokers (26.5%, OR 11.02, 95% CI 2.97, 40.90), and current daily smokers (36.7%, OR 13.90, 95% CI 3.85, 50.22) were all more likely than non-smokers (*i.e.*, those who smoked less than 100 cigarettes in their lifetimes, 3.0%) to try e-cigarette use in order to quit smoking.

3.3. Stopping E-Cigarette Use

Nearly two-thirds (65%) of those who had tried e-cigarettes later discontinued use. People whose main reason for trying e-cigarettes was not goal-oriented were more likely to stop using e-cigarettes than those whose main reason was goal-oriented (81.0% *vs*. 44.7% stopped using, OR 5.28, 95% CI 4.23, 6.58). For example, more than two-thirds of those who tried because of curiosity or the influence of friends or family later discontinued use (69% and 68%, respectively) (Figure 1). However, among participants who tried e-cigarettes in order to quit or cut back on smoking, only 48% discontinued use.

Table 2. Correlates of stopping e-cigarette use (*n* = 2281 former e-cigarette users).

Characteristic	Number Who Stopped Using E-Cigarettes/Total Number in Category (Unweighted *n* and Weighted %)		Bivariate		Multivariate (Includes Bivariate Correlates $p < 0.05$)	
	n	**(%)**	***OR***	**(95% CI)**	***OR***	**(95% CI)**
Overall	2281/3878	(65.2)				
Participant						
Gender						
Male (Ref)	849/1544	(62.7)	1.00	-	1.00	-
Female	1432/2334	(67.7)	1.25	(1.02, 1.54) *	1.23	(1.00, 1.53)
Age						
18–24 (Ref)	306/520	(61.5)	1.00	-		
25–34	473/878	(63.4)	1.08	(0.76, 1.54)		
35–44	337/585	(65.6)	1.20	(0.83, 1.73)		
45–54	475/789	(66.5)	1.24	(0.87, 1.77)		
55–64	442/712	(69.7)	1.44	(1.00, 2.08)		
65 or older	248/394	(66.7)	1.26	(0.80, 1.97)		
Education						
Less than high school (Ref)	118/213	(55.6)	1.00	-	1.00	-
High school	639/995	(69.1)	1.79	(1.17, 2.74) **	1.97	(1.27, 3.05) **
Some college	1086/1837	(64.2)	1.44	(0.95, 2.17)	1.72	(1.12, 2.65) *
Bachelor's degree or higher	438/833	(63.2)	1.37	(0.89, 2.13)	1.66	(1.03, 2.67) *
Smoking status						
Non-smoker (Ref)	126/158	(82.0)	1.00	-	1.00	-
Former smoker	287/392	(82.2)	1.02	(0.51, 2.01)	0.92	(0.46, 1.80)
Current non-daily smoker	225/466	(50.0)	0.22	(0.11, 0.43) ***	0.21	(0.11, 0.40) ***
Current daily smoker	1643/2862	(60.0)	0.33	(0.18, 0.60) ***	0.29	(0.16, 0.52) ***
Race/ethnicity						
Non-Hispanic White (Ref)	1868/3072	(66.3)	1.00	-		
Non-Hispanic Black	136/248	(65.3)	0.96	(0.65, 1.40)		
Non-Hispanic other race or >1 race	138/259	(57.8)	0.69	(0.47, 1.03)		
Hispanic	139/299	(63.1)	0.87	(0.61, 1.25)		

Table 2. *Cont.*

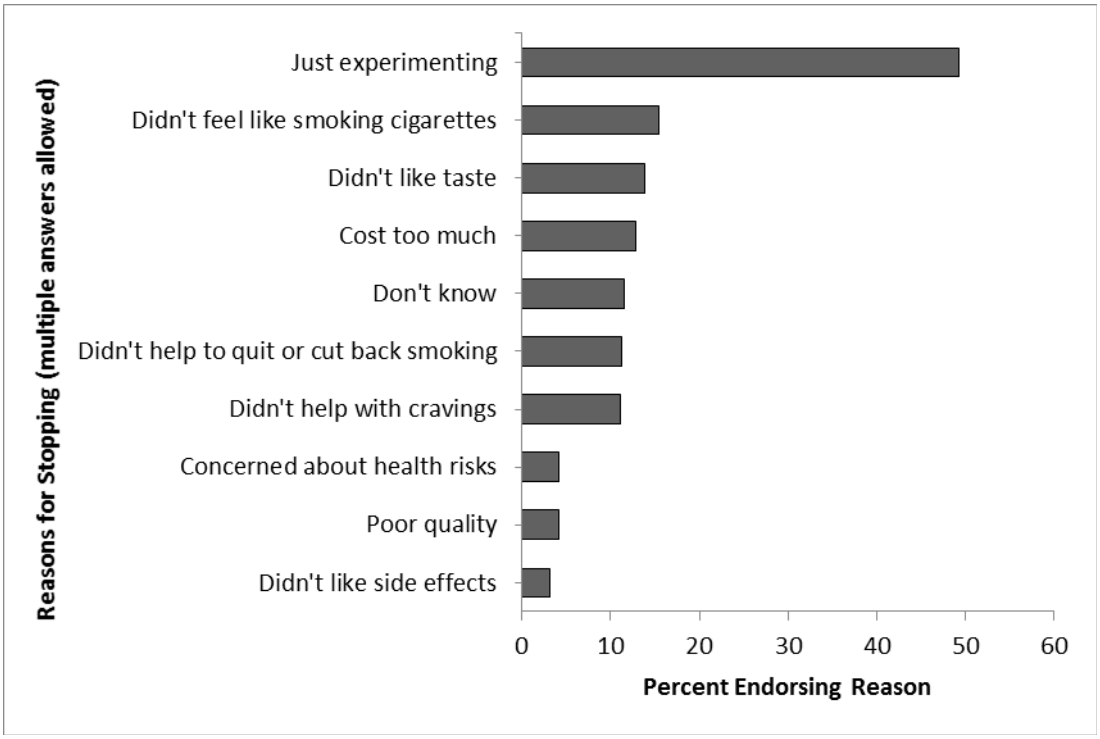
Characteristic	Number Who Stopped Using E-Cigarettes/Total Number in Category (Unweighted *n* and Weighted %)		Bivariate		Multivariate (Includes Bivariate Correlates $p < 0.05$)	
	n	**(%)**	***OR***	**(95% CI)**	***OR***	**(95% CI)**
Marital status						
Married or living with partner (Ref)	1282/2169	(65.6)	1.00	-		
Widowed	82/145	(67.8)	1.10	(0.63, 1.92)		
Divorced or separated	446/714	(69.7)	1.21	(0.90, 1.61)		
Never married	471/850	(61.0)	0.82	(0.63, 1.06)		
Employment						
Working (Ref)	1184/2150	(63.1)	1.00	-	1.00	-
Not working: laid off or looking for work	328/517	(65.4)	1.11	(0.80, 1.52)	1.04	(0.74, 1.49)
Not working: retired, disabled, or other	769/1211	(69.8)	1.35	(1.07, 1.71) *	1.27	(0.98, 1.64)
Household						
Region						
Midwest (Ref)	577/955	(66.0)	1.00	-		
Northeast	339/608	(59.8)	0.77	(0.55, 1.06)		
South	812/1360	(65.7)	0.99	(0.76, 1.28)		
West	553/955	(67.1)	1.05	(0.78, 1.42)		
Annual household income						
Less than $25,000 (Ref)	747/1130	(69.2)	1.00	-	1.00	-
$25,000–$49,999	691/1153	(66.5)	0.88	(0.67, 1.16)	0.89	(0.67, 1.19)
$50,000–$74,999	425/793	(63.8)	0.78	(0.58, 1.06)	0.86	(0.61, 1.20)
$75,000–$99,999	211/415	(60.0)	0.67	(0.47, 0.95) *	0.67	(0.45, 0.99) *
$100,000 or more	207/387	(63.6)	0.77	(0.54, 1.12)	0.69	(0.46, 1.03)

Notes: Multivariate model contains all correlates significant ($p < 0.05$) in bivariate models; OR = odds ratio; CI = confidence interval; Ref = reference category. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The choice to stop using e-cigarettes was associated with education, smoking status, and income in multivariate analysis (Table 2). Compared to those with less than a high school education (56% discontinued), individuals with a high school education (69%, OR 1.97, 95% CI 1.27, 3.05), some college education (64%, OR 1.72, 95% CI 1.12, 2.65), or a Bachelor's degree or higher (63%, OR 1.66, 95% CI 1.03, 2.67) were more likely to stop using e-cigarettes. Current non-daily smokers (50% discontinued, OR 0.21, 95% CI 0.11, 0.40) and current daily smokers (60%, OR 0.29, 95% CI 0.16, 0.52) were less likely than non-smokers (82%) to stop using e-cigarettes, but non-smokers and former smokers did not differ. Finally, participants with annual household incomes between $75,000 and $99,999 were less likely than individuals from households with annual incomes of less than $25,000 to stop using e-cigarettes (60% *vs*. 69%, OR 0.67, 95% CI 0.45, 0.99).

The most common reason for stopping use was that the user was just experimenting with e-cigarettes (49%) (Figure 2). The next most common reasons were that using e-cigarettes did not feel like smoking cigarettes (15%), participants did not like the taste of e-cigarettes (14%), or e-cigarettes cost too much (13%). Just over ten percent of participants stopped using e-cigarettes because they did not help with cravings (11%) or did not help the user quit or cut back on smoking (11%), and 12% stated that they did not know why they stopped using e-cigarettes. In the open-ended question, 3% of former
e-cigarette users said they stopped using e-cigarettes because they quit smoking or quit using nicotine.

Figure 2. Reasons for stopping e-cigarette use (*n* = 2281).



4. Discussion

In this large national study of U.S. adults who had ever used e-cigarettes, the most common reasons for trying e-cigarettes were curiosity, because a friend or family member used, gave, or offered them, and quitting or reducing smoking. Two in three people who tried e-cigarettes later stopped using them,

but this pattern differed by reason for trial. Those whose main reason for trying was goal-oriented had much higher rates of continued use than those who tried for non-goal-oriented reasons like curiosity or the influence of a friend or family member. This pattern suggests there may be distinct subgroups of e-cigarette users with differing experiences of use.

Curiosity, the most common reason for e-cigarette trial, is a natural reason for experimentation. E-cigarettes are a novel product, only introduced in the U.S. market in 2007 [26]. They are widely advertised [27] and covered in the popular media [28–30], both of which help to spread information about and promote experimentation with any innovation [31]. Individuals with higher education tried e-cigarettes because of curiosity more often than those with lower education. Higher education individuals in general are more likely to adopt innovations [31]. In addition, experimentation might be easier in this group because they have greater access to e-cigarettes, which are more available in high socioeconomic status neighborhoods than low socioeconomic status neighborhoods [32]. E-cigarette users also frequently cited the influence of friends or family as a reason for trying the product. Social connections motivate the diffusion of most innovations throughout a population [31]. Smoking behaviors in particular spread through social contacts, partly because similar people gather together in social or family groups [33,34]. Women, who tend to have larger and denser family networks [35] and more intimate friendships [36] than men, were more likely to report the influence of friends or family as a reason for trying e-cigarettes.

In the e-cigarette literature, quitting or reducing smoking is a frequently documented reason for use [7], as we found in this study. Not surprisingly, smokers were more likely than non-smokers to report this as a motivator for trial. Three participants who reported trying e-cigarettes in order to quit smoking met our definition of non-smokers (*i.e.*, smoked less than 100 cigarettes in their lifetime). It is possible that these participants sought to use e-cigarettes in order to curtail their smoking before it became a regular habit. The most common reasons for trial in our study, curiosity and social influence, are less commonly reported in the literature, perhaps because researchers did not allow for these answer choices in closed-ended survey items or because these factors motivated initial trying e-cigarettes but not continued use of e-cigarettes, so they would not be mentioned when asked about reasons for use.

Most respondents who tried e-cigarettes for goal-oriented reasons like smoking cessation or the ability to use e-cigarettes in places where they could not smoke regular cigarettes continued to use them, while those who started for non-goal-oriented reasons like curiosity later stopped using them. We suspect that those who try e-cigarette use for non-goal-oriented reasons have no intention to use them on a regular basis, perhaps because they do not personally identify with the image in their mind of the typical e-cigarette user. Individuals who have positive prototypes of cigarette smokers and identify with those prototypes are more susceptible to smoking [37] and more likely to relapse after quitting [38], and holding more negative beliefs about a typical cigarette smoker is associated with lower interest in trying e-cigarettes [39]. In contrast, only about 20% of those who tried e-cigarettes for goal-directed reasons, most commonly smoking cessation and harm reduction, discontinued use. Value expectancy models of health behavior, such as the Health Belief Model [40], focus on rational, goal-directed behavior. These models describe willingness to engage in health-protective behavior as related to the desire to avoid illness and the belief that this behavior would prevent that illness. Individuals who try e-cigarettes for smoking cessation or harm reduction (the two most common goal-oriented reasons in this study) may be explicitly or implicitly trying to reduce their chances of

developing a smoking related-illness. Among this group, those who then discontinue might do so because the product did not help them achieve their goals (*e.g.*, smoking cessation) or because negative experiences (*e.g.*, bad taste) overrode their goal. Those who continue to use might feel that e-cigarettes are helping them to smoke less or they enjoy the experience of using e-cigarettes once having tried them for cessation reasons.

In general, discontinuation was less common among smokers and more common among those with higher education. This pattern likely reflects the differences in reasons for trial. Individuals with higher education are more likely to try e-cigarettes because of curiosity and then discontinue because they were merely experimenting. Smokers were more likely to try e-cigarettes as a means to quit smoking and then continue to use them for the goal-related reasons described above. After experimentation, the next most common reasons for stopping use were that e-cigarettes did not feel like regular tobacco cigarettes and users did not like their taste. Most e-cigarette users are smokers [7,41,42]. If they are interested in electronic cigarettes as a direct substitute for regular ones, they may be disappointed. Some smokers say that e-cigarettes do not feel like or taste like the "real thing" [22]. Indeed, one frequent response in the open-ended field was that e-cigarettes were "too heavy," presumably in comparison to the weight of regular cigarettes that smokers are accustomed to. In the meantime, e-cigarette technology and design are improving (*e.g.*, stronger batteries, options for user customization), and this trend will likely continue as multinational tobacco corporations invest in the e-cigarette market. Newer models might be more appealing to smokers, and dissatisfaction with feel, taste, or cost may decline. If newer models deliver nicotine more efficiently, fewer smokers might discontinue use because of failure to help manage their cravings. Better products might also be better cessation tools, reducing the number of smokers who stop using because the product fails to help them quit. Alternatively, there may be more smokers who stop using the product because they used it to successfully help them quit nicotine altogether. In the present study, only a small number of respondents reported discontinuing use for that reason.

The present results suggest two distinct groups of e-cigarette users: casual experimenters who almost always stop using and motivated users whose discontinuation decisions relate to the utility or experience of using e-cigarettes. This distinction has implications for research, specifically measurement. Researchers typically report and examine correlates of "ever use" of e-cigarettes. However, "ever users" appear to comprise two different groups. Finer-grained measurement is needed. To distinguish these groups, researchers might assess the number of instances of previous use and rely on a cut-off point for defining ever use as is currently done for established smoking, which is typically defined as having smoked at least 100 cigarettes in a lifetime.

The distinction between casual experimenters and motivated users also has implications for public health practice. If research can identify why certain vulnerable groups are attracted to and start using e-cigarettes (*e.g.*, youth find the candy and fruit flavors appealing), public health campaigns can deliver more effective counter-messages when appropriate. In this sample, the most common reason for trying e-cigarettes was the same among smokers and non-smokers: curiosity. Reducing the appeal of e-cigarettes (*e.g.*, restricting advertising that makes e-cigarettes seem glamorous) or making the product more difficult to obtain might deter non-smokers from trying the product out of mere curiosity.

Ultimately, the extent to which practitioners treat these two groups of e-cigarette users differently will likely depend on whether studies find that e-cigarettes can help smokers quit. If e-cigarettes do not

help smokers quit or have other detrimental effects on public health (e.g., lead to smoking initiation among non-smokers), distinguishing casual experimenters from motivated users could be helpful, as these groups might need different strategies for discouraging use. Alternatively, if future studies show that e-cigarettes are useful cessation tools, practitioners will also need to distinguish between the two types of users. They will need to establish which reasons for trying e-cigarettes are linked to successful smoking cessation. If some reasons for trying (e.g., curiosity, social influence) are not associated with cessation, practitioners should discourage use among individuals who are motivated by those reasons. Should e-cigarettes prove to be useful cessation tools, regulators will also need to ensure that any future policies, such as advertising restrictions, are balanced to ensure that ads can still reach smokers who would use the product as a smoking substitute. In any case, the degree to which e-cigarettes could serve as a harm reduction tool at the population level will depend on the trial and discontinuation choices of different user types.

Limitations to our study include that the TCME survey described e-cigarettes as looking like regular cigarettes, as was the case with first generation models. As e-cigarettes now comprise a class of electronic nicotine delivery devices that do not always look like cigarettes or go by the name "e-cigarettes," some participants might have misidentified themselves as "never users" because the product they had tried was not called an "e-cigarette" or did not look like a cigarette. Measuring the degree of endorsement of different reasons using more than two responses would have allowed finer-grained analysis. In addition, this study was cross-sectional and assessed only reasons for first trial. Thus, participants' recollections of their reasons for first trying e-cigarettes could be subject to recall bias. Further, we could not identify participants who intended to stop using e-cigarettes but had not yet done so and could not examine the reasons for the most recent use among participants who had used e-cigarettes repeatedly. We also could not determine whether stated reasons for trying e-cigarettes prompted later changes in behavior, e.g., whether those who tried e-cigarettes in order to quit smoking actually did so. The study data reflect probability and non-probability samples, but the use of quota sampling and survey weights enabled us to align our estimates with the U.S. population. During survey design, we cognitively tested the survey instrument, but we may have missed some response options for reasons to start and stop using e-cigarettes, such as completely quitting nicotine. The partial overlap between some of the established categories (e.g., a friend or family member uses e-cigarettes) and some of the open-ended responses (e.g., a friend or family member offered a puff of an e-cigarette) suggests that response options could have been more precise. Strengths of the study include the large national sample, extensive formative work, and novel research questions.

5. Conclusions

Few studies have examined reasons for stopping e-cigarette use, despite the fact that most of those who try e-cigarettes discontinue. Understanding these reasons has important public health implications if it helps to segment the population. Not all "ever e-cigarette users" are the same, just as not all cigarette smokers are the same. For example, tobacco control researchers emphasize the need to distinguish between "social smokers" who say they are not addicted to nicotine and those who self-categorize as nicotine-addicted smokers [25]. The extent to which e-cigarette users try the product because they are trying to achieve a goal like smoking cessation appears to be one driver of patterns of

discontinuation. Specifically, those who try for goal-oriented reasons are more likely to continue using, but when they do stop using, they do so for reasons related to product satisfaction. If e-cigarettes are to serve as a harm reduction tool with an overall public health benefit, smokers who try for cessation and health-related reasons will need to be sufficiently satisfied with the product so that they switch completely and not merely experiment with it or engage in dual use. Segmentation between goal-oriented and non-goal-oriented users allows for health interventions to better target users' needs and objectives and thus be more successful [43]. The type of future intervention efforts needed for adult e-cigarette users is uncertain, pending conclusive research on the controversial issue of whether the product can help with smoking cessation. However, even without such data, the need to prevent initiation among youth is clear. Future research should build on this study's findings and explore adolescents' and young adults' reasons for starting and stopping e-cigarette use, as this could help to design programs that prevent e-cigarettes from serving as a gateway to future smoking.

Author Contributions

Jessica K. Pepper, Noel T. Brewer, and Kurt M. Ribisl conceptualized the manuscript's focus and analyses. Sherry L. Emery designed and executed the parent project and assisted with survey item development. Jessica K. Pepper conducted the analyses and drafted the manuscript. Noel T. Brewer, Kurt M. Ribisl, and Sherry L. Emery edited the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Regan, A.K.; Promoff, G.; Dube, S.R.; Arrazola, R. Electronic nicotine delivery systems: Adult use and awareness of the 'e-cigarette' in the USA. *Tob. Control* **2013**, *22*, 19–23.
2. Pepper, J.K.; Emery, S.L.; Ribisl, K.R.; Brewer, N.T. How U.S. adults find out about electronic cigarettes: Implications for public health messages. *Nicotine Tob. Res.* **2014**, *16*, 1140-1144.
3. Emery, S. It's not just Message Exposure Anymore: A New Paradigm for Health Media Research. Presented at Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA, 2013.
4. Kralikova, E.; Novak, J.; West, O.; Kmetova, A.; Hajek, P. Do e-cigarettes have the potential to compete with conventional cigarettes?: A survey of conventional cigarette smokers' experiences with e-cigarettes. *Chest* **2013**, *144*, 1609–1614.
5. Vardavas, C.I.; Filippidis, F.T.; Agaku, I.T. Determinants and prevalence of e-cigarette use throughout the European Union: A secondary analysis of 26 566 youth and adults from 27 Countries. *Tob. Control* **2014**, doi:10.1136/tobaccocontrol-2013-051394.
6. Brown, J.; West, R.; Beard, E.; Michie, S.; Shahab, L.; McNeill, A. Prevalence and characteristics of e-cigarette users in Great Britain: Findings from a general population survey of smokers. *Addict. Behav.* **2014**, *39*, 1120–1125.
7. Pepper, J.K.; Brewer, N.T. Electronic nicotine delivery system (electronic cigarette) awareness, use, reactions, and beliefs: A systematic review. *Tob. Control* **2014**, *23*, 375–384.

8. Goniewicz, M.L.; Knysak, J.; Gawron, M.; Kosmider, L.; Sobczak, A.; Kurek, J.; Prokopowicz, A.; Jablonska-Czapla, M.; Rosik-Dulewska, C.; Havel, C.; *et al*. Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. *Tob. Control* **2014**, *23*, 133–139.
9. Kosmider, L.; Sobczak, A.; Fik, M.; Knysak, J.; Zaciera, M.; Kurek, J.; Goniewicz, M.L. Carbonyl compounds in electronic cigarette vapors: Effects of nicotine solvent and battery output voltage. *Nicotine Tob. Res.* **2014**, *16*, 1319–1326.
10. Cheng, T. Chemical evaluation of electronic cigarettes. *Tob. Control* **2014**, *23*, ii11–ii17.
11. Czogala, J.; Goniewicz, M.L.; Fidelus, B.; Zielinska-Danch, W.; Travers, M.J.; Sobczak, A. Secondhand exposure to vapors from electronic cigarettes. *Nicotine Tob. Res.* **2014**, *16*, 655–662.
12. Bhatnagar, A.; Whitsel, L.P.; Ribisl, K.M.; Bullen, C.; Chaloupka, F.; Piano, M.R.; Robertson, R.M.; McAuley, T.; Goff, D.; Benowitz, N.; *et al.* Electronic cigarettes: A policy statement from the American Heart Association. *Circulation* **2014**, doi:10.1161/CIR.0000000000000107.
13. Grana, R.; Benowitz, N.; Glantz, S.A. E-cigarettes: A scientific review. *Circulation* **2014**, *129*, 1972–1986.
14. Adkison, S.E.; O'Connor, R.J.; Bansal-Travers, M.; Hyland, A.; Borland, R.; Yong, H.H.; Cummings, K.M.; McNeill, A.; Thrasher, J.F.; Hammond, D.; *et al*. Electronic nicotine delivery systems: International Tobacco Control Four-Country Survey. *Am. J. Prev. Med.* **2013**, *44*, 207–215.
15. Bullen, C.; Howe, C.; Laugesen, M.; McRobbie, H.; Parag, V.; Williman, J.; Walker, N. Electronic cigarettes for smoking cessation: A randomised controlled trial. *Lancet* **2013**, *382*, 1629–1637.
16. Brown, J.; Beard, E.; Kotz, D.; Michie, S.; West, R. Real-world effectiveness of e-cigarettes when used to aid smoking cessation: A cross-sectional population study. *Addiction* **2014**, doi:10.1111/add.12623.
17. Grana, R.A.; Popova, L.; Ling, P.M. A longitudinal analysis of electronic cigarette use and smoking cessation. *JAMA Intern. Med.* **2014**, *174*, 812–813.
18. Vickerman, K.A.; Carpenter, K.M.; Altman, T.; Nash, C.M.; Zbikowski, S.M. Use of electronic cigarettes among state tobacco cessation quitline callers. *Nicotine Tob. Res.* **2013**, *15*, 1787–1791.
19. Dockrell, M.; Morison, R.; Bauld, L.; McNeill, A. E-cigarettes: Prevalence and attitudes in Great Britain. *Nicotine Tob. Res.* **2013**, *15*, 1737–1744.
20. Etter, J.F.; Bullen, C. Electronic cigarette: Users profile, utilization, satisfaction and perceived efficacy. *Addiction* **2011**, *106*, 2017–2028.
21. Foulds, J.; Veldheer, S.; Berg, A. Electronic cigarettes (e-cigs): Views of aficionados and clinical/public health perspectives. *Int. J. Clin. Pract.* **2011**, *65*, 1037–1042.
22. Etter, J.F. Electronic cigarettes: A survey of users. *BMC Public Health* **2010**, *10*, 231.
23. Farsalinos, K.E.; Romagna, G.; Tsiapras, D.; Kyrzopoulos, S.; Spyrou, A.; Voudris, V. Impact of flavour variability on electronic cigarette use experience: An internet survey. *Int. J. Environ. Res. Public Health* **2013**, *10*, 7272–7282.

24. Kralikova, E.; Kubatova, S.; Truneckova, K.; Kmetova, A.; Hajek, P. The electronic cigarette: What proportion of smokers have tried it and how many use it regularly? *Addiction* **2012**, *107*, 1528–1529.
25. Schane, R.E.; Glantz, S.A.; Ling, P.M. Nondaily and social smoking: An increasingly prevalent pattern. *Arch. Intern. Med.* **2009**, *169*, 1742–1744.
26. Noel, J.K.; Rees, V.W.; Connolly, G.N. Electronic cigarettes: A new 'tobacco' industry? *Tob. Control* **2011**, *20*, doi:10.1136/tc.2010.038562.
27. Kornfield, R.; Huang, J.; Vera, L.; Emery, S.L. Rapidly increasing promotional expenditures for e-cigarettes. *Tob. Control* **2014**, doi:10.1136/tobaccocontrol-2014-051580.
28. Richtel, M. E-Cigarettes, by Other Names, Lure Young and Worry Experts. *The New York Times*, 5 March 2014.
29. Rooke, C.; Amos, A. News media representations of electronic cigarettes: An analysis of newspaper coverage in the UK and Scotland. *Tob. Control* **2013**, doi:10.1136/tobaccocontrol-2013-051043.
30. Rayman, N. Scientists to WHO: Don't Classify E-Cigs as Tobacco Products. *Time Magazine*, 29 May 2014.
31. Rogers, E.M. *Diffusion of Innovations*; 5th ed.; Free Press: New York, NY, USA, 2003.
32. Rose, S.W.; Barker, D.C.; D'Angelo, H.; Khan, T.; Huang, J.; Chaloupka, F.J.; Ribisl, K.M. The availability of electronic cigarettes in US retail outlets, 2012: Results of two national studies. *Tob. Control* **2014**, *23* (Suppl. 3), iii10–iii16.
33. Flatt, J.D.; Agimi, Y.; Albert, S.M. Homophily and health behavior in social networks of older adults. *Fam. Community Health* **2012**, *35*, 312–321.
34. Christakis, N.A.; Fowler, J.H. The collective dynamics of smoking in a large social network. *N. Engl. J. Med.* **2008**, *358*, 2249–2258.
35. Moore, G. Structural determinants of men's and women's personal networks. *Am. Sociol. Rev.* **1990**, *55*, 726–735.
36. Booth, A. Sex and social participation. *Am. Sociol. Rev.* **1972**, 183–193.
37. McCool, J.P.; Cameron, L.; Petrie, K. Stereotyping the smoker: Adolescents' appraisals of smokers in film. *Tob. Control* **2004**, *13*, 308–314.
38. Gibbons, F.X.; Eggleston, T.J. Smoker networks and the "typical smoker": A prospective analysis of smoking cessation. *Health Psychol.* **1996**, *15*, 469–477.
39. Pepper, J.K.; Reiter, P.L.; McRee, A.L.; Cameron, L.D.; Gilkey, M.B.; Brewer, N.T. Adolescent males' awareness of and willingness to try electronic cigarettes. *J. Adolesc. Health* **2013**, *52*, 144–150.
40. Rosenstock, I.M. The Health Belief Model and preventive health behavior. *Health Educ. Behav.* **1974**, *2*, 354–386.
41. Zhu, S.H.; Gamst, A.; Lee, M.; Cummins, S.; Yin, L.; Zoref, L. The use and perception of electronic cigarettes and snus among the U.S. population. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0079332.

42. Pearson, J.L.; Richardson, A.; Niaura, R.S.; Vallone, D.M.; Abrams, D.B. E-cigarette awareness, use, and harm perceptions in US adults. *Am. J. Public Health* **2012**, *102*, 1758–1766.
43. Kreuter, M.W.; Wray, R.J. Tailored and targeted health communication: Strategies for enhancing information relevance. *Am. J. Health Behav.* **2003**, *27* (Suppl. 3), S227–S232.

HHS Public Access

Author manuscript

Addiction. Author manuscript; available in PMC 2015 September 10.

Published in final edited form as:

Addiction. 2010 February ; 105(2): 343–355. doi:10.1111/j.1360-0443.2009.02780.x.

Reduced nicotine content cigarettes: effects on toxicant exposure, dependence and cessation

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Abstract

Aims—To examine the effects of reduced nicotine cigarettes on smoking behavior, toxicant exposure, dependence and abstinence.

Design—Randomized, parallel arm, semi-blinded study.

Setting—University of Minnesota Tobacco Use Research Center.

Interventions—Six weeks of: (i) 0.05 mg nicotine yield cigarettes; (ii) 0.3 mg nicotine yield cigarettes; or (iii) 4 mg nicotine lozenge; 6 weeks of follow-up.

Measurements—Compensatory smoking behavior, biomarkers of exposure, tobacco dependence, tobacco withdrawal and abstinence rate.

Findings—Unlike the 0.3 mg cigarettes, 0.05 mg cigarettes were not associated with compensatory smoking behaviors. Furthermore, the 0.05 mg cigarettes and nicotine lozenge were associated with reduced carcinogen exposure, nicotine dependence and product withdrawal scores. The 0.05 mg cigarette was associated with greater relief of withdrawal from usual brand cigarettes than the nicotine lozenge. The 0.05 mg cigarette led to a significantly higher rate of cessation than the 0.3 mg cigarette and a similar rate as nicotine lozenge.

Conclusion—The 0.05 mg nicotine yield cigarettes may be a tobacco product that can facilitate cessation; however, future research is clearly needed to support these preliminary findings.

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Clinical trial registration number
NCT 00777569.

Declarations of interest

Peter Shields has served as an expert witness on behalf of plaintiffs in litigation cases against tobacco companies and Stephen Hecht is an expert witness for the plaintiff in 'Kelly Hill *et al.* v. U.S. Smokeless Tobacco Company'. Dorothy Hatsukami has consulted for Pfizer, Abbott Laboratories and Novartis (travel expenses only) and has received a research grant from Nabi Biopharmaceuticals.

Keywords

Biomarkers of exposure; compensatory smoking; nicotine dependence; reduced nicotine cigarettes; tobacco cessation; tobacco withdrawal

INTRODUCTION

In recent years, tobacco companies have renewed their efforts to manufacture and market potential reduced exposure tobacco products (called PREPs) to cigarette smokers. These products include cigarettes modified to reduce toxicants but maintain levels of nicotine [1,2]. However, to date these modified cigarettes have not shown great promise for reducing exposure to toxicants significantly [3,4]. Alternatively, cigarettes with significantly reduced nicotine (the major known addictive constituent in cigarettes) may have promise in dramatically reducing cigarette use [5]. Unlike ‘light’ or ‘mild’ cigarettes that reduce nicotine yields through filter ventilation but which lead to similar levels of cotinine and toxicants as regular cigarettes due to compensatory smoking behavior [6–8], reduced nicotine in cigarette tobacco makes compensatory smoking more difficult. Limited data from previous studies of such products suggest that compensatory smoking does not occur, toxicant exposure does not increase and abstinence may be facilitated [9,10]. Theoretically, reducing levels of nicotine to the point of non-reinforcement would lead to extinction or cessation of smoking as well as unlearning cues associated with reinforcement.

No clinical trial has examined the effects of smoking reduced nicotine cigarettes on smoking behavior, on resulting toxicant exposure, on withdrawal symptoms and craving, on dependence scores or on abstinence rates compared with medicinal nicotine products.

To address these questions, we conducted a study in which smokers were randomized to 6 weeks of 0.3 mg nicotine yield cigarettes, 0.05 mg nicotine yield cigarettes or to 4 mg medicinal nicotine lozenges. Medicinal nicotine has been recommended as the comparator to which PREPs should be tested for toxicant exposure [11] and also serves as a usual care condition to compare abstinence rates across products. As primary outcomes, we hypothesized that smoking behavior, toxicant exposure, withdrawal and craving upon product discontinuation and product dependence would be relatively less for the 0.05 mg nicotine yield cigarette compared to the 0.3 mg nicotine yield cigarette, because the 0.05 mg cigarette would lead to less reinforcement from smoking than the 0.3 mg cigarette. As a secondary outcome, we hypothesized that pre-treatment with 0.05 mg cigarettes will produce favorable quit rates that are similar to nicotine lozenges and better than 0.3 mg cigarettes.

Our goal was to examine the feasibility of using these cigarettes as a method to reduce smoking behavior significantly and as a potential cessation tool, which would lead subsequently to reduction in harm. The results also provide information on the role that different aspects of tobacco use (nicotine versus sensory aspects of smoking) contributes to tobacco addiction.

METHODS

Subjects

Smokers of 'light' cigarettes (0.7–1.0 mg nicotine/cigarette) between the ages of 18 and 70 years who were interested in quitting smoking were recruited via advertisement. To be eligible smokers had to (i) have smoked 10–40 cigarettes daily for the past year (the range was instituted to reduce heterogeneity); (ii) be in good physical health; (iii) be in good psychiatric health; and (iv) have no contraindications for medicinal nicotine use. Subjects using other tobacco or nicotine products were excluded, as were subjects who were pregnant or nursing.

Study design

After a telephone screening to determine preliminary eligibility, an orientation session was held at which the study was explained further, written informed consent was obtained and a more thorough screening for eligibility was performed.

After a 2-week period during which baseline measurements were collected while subjects smoked *ad libitum*, subjects were assigned to one of three conditions: (i) 0.3 mg nicotine yield cigarettes, (ii) 0.05 mg nicotine yield cigarettes or (iii) nicotine lozenges (4 mg). Quest cigarettes (manufactured by Vector; Vector Tobacco Inc., Durham, NC, USA) were chosen because they are commercially available reduced nicotine cigarettes (nicotine yield as measured in mainstream smoke by the Federal Trade Commission method) with reduced levels of tobacco-specific carcinogens compared to conventional cigarettes [12]. Subjects assigned to the cigarette conditions were blinded as to which cigarette they received (i.e. 0.05 mg versus 0.3 mg). Subjects were instructed to use their assigned treatment for 6 weeks (after which time they were to discontinue product use) and to not use other nicotine or tobacco products during the treatment or any products during the follow-up period. Subjects were seen weekly during the 6-week treatment period and at 1, 2, 4 and 6 weeks after cessation. Subjects who completed the study were paid up to $345.

To allow for compensatory smoking, at each visit subjects assigned to either cigarette condition were provided a supply equivalent to 150% of their baseline smoking rate and were told to smoke *ad libitum*. Subjects assigned to receive the 4 mg nicotine lozenge were asked to quit smoking and to use at least six to eight pieces per day, the mean number of lozenges used among smokers enrolled in a clinical trial [13]. If side effects suggested that the dose was too high, the 2 mg nicotine lozenge was substituted at that time. Subjects maintained a daily smoking diary in which they recorded any cigarettes smoked (either those assigned to them or their own). If they smoked cigarettes other than those assigned, they were to note when that cigarette was smoked. They were not penalized for smoking that cigarette, but told that although we do not encourage them to smoke cigarettes other than those assigned, it is crucial to the study that they indicate to us whenever they smoked any other cigarettes.

Brief (approximately 10 minutes) standardized counseling was provided at each of the visits during the treatment phase of the study. Subjects assigned to the cigarette conditions were counseled to consider the use of these products as a step towards quitting. They discussed

any difficulties they experienced with switching cigarettes and behavioral strategies to resist smoking other (non-Quest) cigarettes. Subjects assigned to the nicotine lozenge condition were provided with treatment tools recommended by the US Clinical Practice Guideline [14]. During the abstinence phase, all subjects received counseling similar to that received by the subjects assigned to the nicotine lozenge condition. Therefore, all three treatment groups received similar amounts of behavioral support.

Outcome measures

Biomarkers of tobacco toxicant exposure measures included: (i) urinary cotinine plus cotinine–glucuronide (total cotinine), a direct measure of nicotine exposure (product nicotine delivery and amount consumed) [15,16]; (ii) alveolar carbon monoxide (CO) measured using the Bedfont Micro Smokerlyzer® (Bedfont Scientific Limited, Kent, UK); (iii) urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides (total NNAL), metabolites of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone ((NNK; [17]); (iv) urinary *N*′-nitrosonornicotine and its glucuronide (total NNN), metabolites of the tobacco-specific carcinogen *N*′-nitrosonornicotine [18]; (v) urinary 1-hydroxypyrene and its glucuronide and sulfate (total 1-HOP), a metabolite of pyrene which is an accepted biomarker for uptake of carcinogenic polycyclic aromatic hydrocarbons (PAH; [19]); (vi) urinary 3-hydroxypropylmercapturic acid (3-HPMA), a metabolite of the toxicant, acrolein [20]; and (vii) *S*-phenylmercapturic acid (S-PMA), a metabolite of the human leukemogen, benzene [21]. These biomarkers reflect exposure to particulate or smoke constituents in cigarettes. All measures were assessed at baseline. Additionally, carbon monoxide was assessed at each treatment clinic visit, cotinine at weeks 2 and 6 of treatment and at follow-up visits (except at 1 week post-treatment) and biomarkers for other exposure measures at weeks 2 and 6 of treatment. CO, 1-HOP, 3-HPMA, S-PMA are influenced by factors other than tobacco while total cotinine, total NNAL and total NNN are tobacco-specific.

Subjective measures included: (i) a tobacco use questionnaire that asked about current tobacco use status (cigarettes and other tobacco products), number of ≥24-hour quit attempts and duration of abstinence during these quit attempts; (ii) a daily diary detailing the number of cigarettes smoked; (iii) the Minnesota Nicotine Withdrawal Scale, a widely used scale that assesses withdrawal from cigarettes [22–24], nicotine gum [25,26] and smokeless tobacco [25,27]; (iv) the Fagerstrom Test for Nicotine Dependence (FTND, [28]), the most widely used and psychometrically tested scale for nicotine dependence; and (v) perceived health risk, a ladder involving rating risk for addiction of a product on a scale ranging from 1 to 10. All these measures were assessed at baseline. Cigarette or product use was assessed daily, the tobacco use questionnaire and Minnesota Nicotine Withdrawal Scale at each clinic visit, and the FTND and perceived health risk at weeks 2 and 6.

This study was approved by the University of Minnesota Research Subjects Protection Programs Institutional Review Board.

Statistical analysis

Subjects' baseline characteristics including demographics and smoking history were compared among three treatment groups. Discrete variables were analyzed using Pearson's χ^2 test or Fisher's exact test. Continuous variables were analyzed using either one-way analysis of variance (ANOVA) or Kruskall–Wallis test.

For outcome variables measured at each baseline visit, the average was used as the baseline measurement. Repeated-measures ANOVA was used for outcomes that had been measured repeatedly from baseline to the end of the treatment phase. Each repeated-measures ANOVA model contained five terms: treatment effect, visit effect, interaction effect between treatment and visit, random subject effect (between-subject error) and random error (within-subject error). The variance–covariance structure was specified as the first-order autoregression, and variance parameters were estimated using restricted maximum likelihood method with Satterthwaite approximation. The *P*-values reported for multiple comparisons were unadjusted. Biomarkers including cotinine, NNN, NNAL, 1-HOP, 3-HPMA and S-PMA were analyzed in a natural log scale for repeated-measures ANOVA such that the model assumptions of normality and equal variances can hold, and geometric means in original units were also calculated. The differences in the point prevalence (no smoking in past 7 days) and continuous abstinence (no smoking in past 4 weeks) rates during the follow-up period between treatment groups were evaluated using χ^2 tests, as were dropout rates. SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) was used. A *P*-value <0.05 indicated statistical significance.

RESULTS

Subjects

Of 883 subjects screened over the telephone, 462 were considered eligible for participation. Primary reasons for ineligibility were smoking outside the range of eligible cigarettes per day, smoking ineligible cigarettes or unstable illness. Two hundred and twenty-eight attended the orientation meeting, 225 signed the informed consent form and 165 were assigned randomly to treatment (53 to 0.05 mg cigarettes; 52 to 0.3 mg cigarettes and 60 to nicotine lozenges). Dropout rates throughout the study were highest in those assigned to nicotine lozenges and lowest in those assigned to 0.3 mg nicotine yield cigarettes, with significant differences observed between groups at the end of the 6-week treatment period (48.3% dropout rate for nicotine lozenge versus 39.6% for 0.05 mg nicotine cigarettes versus 25.0% for 0.3 mg nicotine cigarettes; $\chi^2 = 6.49$, $P = 0.0389$). Figure 1 illustrates the number of dropouts in each group at various stages throughout the study with reasons for dropouts indicated. The demographics and smoking history of smokers are shown in Table 1, with no significant differences among the experimental groups except age of becoming a regular smoker ($P = 0.0195$). There were no significant differences in demographics between subjects who dropped out of the study after randomization and those who completed the entire study.

Product use during treatment

The number of assigned cigarettes smoked per day during the treatment period is illustrated in Fig. 2a. Significant treatment ($F_{(2, 180)} = 102.48$, $P < 0.0001$), time ($F_{(6, 648)} = 37.77$, $P < 0.0001$) and treatment × time ($F_{(12, 648)} = 62.38$, $P < 0.0001$) effects were observed. In those smoking 0.3 mg cigarettes, the number of cigarettes smoked per day increased significantly ($P = 0.0127$ to $P < 0.0001$) at each of the first 5 weeks of treatment compared to the number of usual brand cigarettes they were smoking at baseline (Fig. 2a). This is in contrast to the significantly decreased ($P = 0.0043$ to $P < 0.0001$) number of cigarettes smoked per day (relative to baseline) observed after week 2 in those assigned 0.05 mg cigarettes. At week 6, the mean number of 0.3 mg cigarettes smoked per day was significantly greater than that of 0.05 mg cigarettes smoked ($t = 4.73$, $P < 0.0001$). Nicotine lozenge use (among those assigned to this condition) remained relatively stable throughout the 6-week treatment period and ranged from a mean of 5.9 [standard deviation (SD = 2.4] lozenges per day at week 6 to a mean of 6.9 (SD = 3.5) lozenges per day at week 3.

Of those subjects who had not dropped out at the visit in question, subjects were most likely to use a nicotine or tobacco product other than what was assigned to them during the first week of treatment with 30.4% of those assigned to 0.05 cigarettes, 22.9% of those assigned to 0.3 mg and 40.8% assigned to nicotine lozenges reporting such use. After week 1, the percentage who reported using a non-study-assigned nicotine or tobacco product ranged from 0 to 12.5% (during weeks 2–6) in the 0.05 mg cigarette group, 5.0 to 10.5% in the 0.3 mg cigarette group and 8.3 to 21.9% in the nicotine lozenge group. No significant difference between groups was observed in the percentage of subjects using tobacco or nicotine products other than those assigned at any weekly visit except for week 6. At the week 6 visit, 5.3% in the 0.05 mg cigarette group, 0% in the 0.3 mg cigarette group and 21.9% in the nicotine lozenge group ($P = 0.0056$) reported using such products. Among those reporting smoking usual brand cigarettes during the treatment period, the mean number of cigarettes smoked ranged from 0.3 to at most 6.4 per week. Distribution of cotinine concentrations at week 6 demonstrates that, in the 0.05 mg nicotine cigarette group, very few individuals had substantial cotinine concentrations, suggesting that most did not use non-study nicotine-containing products during the study. The distribution shows that 22 subjects had cotinine concentrations between 0 and 250 ng/ml, four between 251 and 500 ng/ml, three between 501 and 1000 ng/ml and three had >5000 ng/ml.

Effects of products on biomarkers of exposure during treatment

Biomarker concentrations during the treatment period for exhaled CO are illustrated in Fig. 2b. Urinary total cotinine, total NNAL, total NNN, total 1-HOP, 3-HPMA and S-PMA are presented in Table 2.

For all seven biomarkers of exposure, significant treatment effects (P-values 0.0131–<0.0001), time effects (P-values 0.0001–<0.0001) and treatment × time interaction effects (P-values 0.0045–<0.0001) were found.

As illustrated in Fig. 2b, exhaled CO concentrations followed a similar pattern as seen for number of cigarettes smoked per day. Exhaled CO concentrations increased during the first

5 weeks of treatment in those using 0.3 mg cigarettes, whereas in those receiving 0.05 mg cigarettes exhaled CO decreased gradually, with a statistically significant decrease observed at week 6 of treatment when compared with baseline ($P = 0.0247$). At week 6, exhaled CO concentrations were nearly significantly different ($P = 0.0569$) between the two cigarette groups. Urinary cotinine concentrations decreased significantly in all treatment groups, with the greatest decrease observed in the 0.05 mg cigarette group and moderate decreases occurring in the 0.3 mg cigarette and nicotine lozenge groups (Table 2). For the other biomarkers assessed, greatest decreases from baseline were found in the group receiving nicotine lozenge with the smallest changes in biomarker concentrations observed mainly in the group receiving 0.3 mg cigarettes (Table 2).

Effects of products on subjective responses during treatment

Dependence—FTND score and perceived risk for addiction score during treatment are illustrated in Fig. 3a,b. Both these measures showed significant treatment ($P = 0.0124$ and <0.0001, respectively), time (P-values <0.0001) and treatment × time interaction (P-values <0.0001) effects. Significant decreases in FTND and perceived risk of addiction scores were observed for the 0.05 mg cigarette and nicotine lozenge groups (all P-values ≤ 0.001) at week 6 compared to baseline. For the 0.3 mg cigarette group, perceived risk of addiction decreased significantly between baseline and week 6 ($P < 0.0001$); however, FTND score did not ($P = 0.4810$). At week 6, significant differences between groups were found in FTND (P-values 0.0315–<0.0001) and perceived risk of addiction (P-values 0.0456–<0.0001), with the highest levels observed in those assigned to 0.3 mg cigarettes and the lowest in those assigned to nicotine lozenge.

Nicotine craving and withdrawal symptoms during treatment and follow-up are illustrated in Fig. 3c,d. Nicotine craving and withdrawal symptoms at the time of switching to products (week 1) and cessation from products (week 7) were examined. Upon cessation of usual brand cigarettes and switching to the products, there was a significant increase in withdrawal symptoms (P-values 0.0188–<0.0001) and no significant change in craving in all three treatment groups. Increase in nicotine withdrawal scores upon cessation of usual brand cigarettes (week 1 compared to baseline) was significantly smaller for the group assigned to 0.05 mg cigarettes compared to the group assigned nicotine lozenges ($P = 0.0253$) and nearly significantly smaller ($P = 0.0917$) than for those assigned to 0.3 mg cigarettes. Upon cessation of the product (week 7 compared to week 6), significant increase in craving ($P = 0.0079$) and withdrawal symptoms ($P < 0.0001$) were observed for the 0.3 mg cigarette group. In those discontinuing 0.05 mg cigarettes, craving increased significantly ($P = 0.0138$), but withdrawal symptoms did not ($P = 0.2297$). In those discontinuing nicotine lozenge, neither changes in craving ($P = 0.0814$) nor withdrawal symptoms ($P = 0.4856$) were increased significantly. Change in withdrawal symptoms was significantly lower in those discontinuing 0.05 mg cigarettes ($P = 0.0006$) or nicotine lozenges ($P = 0.0002$) compared to those discontinuing 0.3 mg cigarettes, with no significant differences in craving observed between groups.

Abstinence

Abstinence rates were calculated using the intent-to-treat sample (at the point of random assignment to the product and before baseline measures). Dropouts were considered treatment failures. Biochemically verified [CO < 8 parts per million (p.p.m.)] point prevalence rates of abstinence from cigarettes at each of the follow-up visits and 4-week continuous abstinence rates are shown in Table 3. In this analysis, subjects were allowed to use lozenges. Similarly, biochemically verified (CO < 8 p.p.m. and total cotinine <35 ng/ml) abstinence from all nicotine-containing products (including lozenges) is also listed in Table 3. For abstinence from cigarettes, 4-week continuous abstinence rates were highest in those receiving 0.05 mg nicotine cigarettes and lowest in those receiving 0.3 mg nicotine cigarettes, with the difference across the three groups nearly significant (Table 3). Point prevalence abstinence rates followed a similar pattern, with significant differences between groups observed in abstinence rates verified by both CO and urinary cotinine concentrations. CO verified point prevalence abstinence rates were statistically significant only at the week 6 post-treatment visit and nearly significant at the weeks 2 and 4 post-treatment visits.

Unlike the cigarette conditions, the nicotine lozenge condition involved complete cessation from cigarettes from the onset of treatment, providing a potentially unfair advantage to the cigarette conditions. Therefore, to determine if duration of cigarette abstinence had an impact on abstinence rates in the nicotine lozenge condition, the continuous CO verified abstinence rates from the last 4 weeks of product use without smoking was compared with the 4-week continuous CO verified abstinence rates at the end of the follow-up period. The results were identical (35%). In addition, the point prevalence rate at the end of 6 weeks of product use was compared with the rate at the end of the follow-up period and the rates were similar (40.0% versus 36.7%).

DISCUSSION

This study showed that, unlike the 0.3 mg nicotine yield cigarettes, 0.05 mg nicotine yield cigarettes were not associated with compensatory smoking behavior. Thus, although increased smoking and exhaled CO were observed with the 0.3 mg cigarettes, decreases in cigarette intake and eventually in exhaled CO were observed for the 0.05 mg cigarettes, a finding similar to another study [29]. The 0.05 mg cigarettes were also associated with reduced exposure biomarker levels (e.g. total NNAL, total NNN, 3-HPMA, S-PMA), reduced nicotine dependence and withdrawal scores. Conversely, the 0.3 mg cigarettes did not result in significant decreases in most exposure biomarkers, led to persistent self-reported dependence and higher levels of withdrawal from this product compared to the other products. As expected, nicotine lozenge was associated with the most consistent reductions in toxicant exposure, dependence on cigarettes and perceived risk for addiction. It is important to note that levels of total 1-HOP, 3-HPMA, and SPMA did not attain zero values for the nicotine lozenge group because there are environmental and endogenous sources of pyrene, acrolein and benzene other than tobacco smoke exposure. [30] Furthermore, total NNAL has a long half-life [31] and there is evidence for endogenous formation of NNN is some users of nicotine replacement therapy [32] In addition, the results showed that a few subjects in the nicotine lozenge group had reported using cigarettes or

other non-assigned tobacco products during the first 6 weeks (8.3–21.9%). Interestingly, the 0.05 mg cigarette led to the highest abstinence rates of the three products tested, although difference in continuous abstinence rates did not reach statistical significance.

The slope of the decline in cigarette smoking rate was slightly faster than the decline in exhaled CO concentrations for the 0.05 mg condition. This result may reflect the use of usual cigarettes among some subjects during the first few weeks of treatment, or may reflect subjects' engagement in some compensatory smoking behavior during the initial period of adjustment to the product. None the less, CO decreased over time, as the number of cigarettes smoked decreased and the minimal compensatory smoking observed when subjects were converted to the 0.05 mg cigarettes is consistent with the results of several small studies in which limited or no compensatory smoking was found when subjects smoked either a single reduced nicotine cigarette in a laboratory setting or smoked one of five progressively lower nicotine content cigarettes for a week [9,10]. Conversely, studies examining use of highly ventilated low-yield cigarettes have found that substantial compensation occurs [33].

The reduction in levels of urinary total NNAL and NNN is consistent with reduced tobacco specific nitrosamine levels found in these products [12]. For example, Marlboro and Camel 'light' cigarettes have NNK levels of 0.68 and 0.55 μg/g wet weight and NNN levels of 2.8 and 2.7 μg/g wet weight, respectively, while the corresponding values for the 0.3 mg and 0.05 mg Quest products were 0.19 and 0.054 μg/g wet weight NNK and 0.82 and 0.83 μg/g wet weight NNN. For the 0.05 mg cigarettes, additional reduction in carcinogen and toxicant exposures (to acrolein and benzene) is probably attributable to the observed reduction in cigarette intake such that, by the end of treatment, most biomarker levels in this group were not significantly different from those in the nicotine lozenge group. Therefore, the observed reduction in biomarker levels is due probably to differences in the amount of the constituents related to the biomarkers in the product itself and in the case of the cigarettes, the amount of product use. On the other hand, no reductions were observed for 1-HOP, which may indicate that exposure to polycyclic aromatic hydrocarbons may be significant when using both the 0.3 mg and 0.05 mg cigarettes and not affected by the degree of reduction in smoking behavior observed in this study.

Although blind to the nicotine content of their assigned cigarettes, only smokers in the 0.05 mg group appeared to experience a reduction on a scale measuring nicotine dependence. However, the perceived risk for addiction decreased for both cigarette products. The reduced nicotine dependence associated with the 0.05 mg cigarettes is consistent with other studies which show reduced FTND scores [9] or decreased motivation to smoke [29] after smoking low nicotine content or denicotinized cigarettes. Another indicator of reduced dependence is the reduction in withdrawal symptoms experienced after cessation from the 0.05 mg cigarettes and the nicotine lozenge compared to withdrawal from 0.3 mg cigarettes. It is notable that, although the 0.3 mg cigarettes and nicotine lozenges were associated with similar cotinine levels, less withdrawal was observed after nicotine lozenge discontinuation. This suggests that withdrawal may be affected by the nicotine pharmacokinetics of the discontinued product. On the other hand, craving increased for both 0.3 mg and 0.05 mg cigarette conditions after cessation of these products, but not for nicotine lozenge. This

finding would indicate that craving for cigarettes has a different abstinence pattern than total withdrawal [34] and may be affected by different aspects of smoking (e.g. missing the sensory aspects of smoking as opposed to primarily nicotine).

Our finding that use of 0.05 mg cigarettes led to greater withdrawal symptom relief than use of nicotine lozenge and no difference than the relief with use of 0.3 mg cigarettes suggests further that non-nicotine components of cigarette dependence (e.g. other tobacco constituents, sensory aspects of smoking) contribute to the relief of withdrawal symptoms. On the other hand, all products appeared to relieve craving equally. These findings are consistent with other studies demonstrating that use of denicotinized cigarettes reduce craving, negative affect and in some studies, withdrawal symptoms or a subset of symptoms during periods of short-term abstinence [9,35–46]. The use of 0.05 mg nicotine cigarettes, by reducing dependence and withdrawal symptoms, may therefore be a promising tool for achieving smoking cessation. Indeed, our study demonstrated that smoking cessation rates in those receiving 0.05 mg cigarettes were equivalent to (if not slightly higher than) cessation rates in those receiving nicotine lozenges. A study by Benowitz *et al.* found that 4 weeks following the end of a progressive reduction in nicotine content of cigarettes 20% of subjects attained abstinence [9]. This rate is surprisingly high, given that these subjects were not enrolling in a cessation study.

Our study suggests that significantly reducing nicotine content of cigarettes may facilitate abstinence by making smoking cessation easier to achieve. For the subpopulation of smokers who rely on nicotine for self-medication the use of medicinal products, either in its current form or in a form that results in faster delivery, greater amounts or in other ways that are more satisfying could be considered [47]. This approach is supported by several prior studies suggesting that the use of denicotinized cigarettes in combination with nicotine patch for smoking cessation show promise [48–50].

A major limitation of the current study was the large number of dropouts. About a third to almost half the population dropped out before the end of follow-up and about one-fifth to more than a third dropped out during treatment, with dropout rates lowest in the 0.3 mg cigarette group and highest in the nicotine lozenge group. Another limitation was the inability to determine if smokers were compliant with the study procedures (i.e. that they used the assigned products solely), although the observed cotinine levels are generally consistent with what would be expected with each product (i.e. larger decreases in the 0.05 mg nicotine cigarette group than the other two groups). Despite the fact that the data may be contaminated by smokers who smoked usual brand cigarettes during intervention, the results show that the 0.05 mg cigarette does not lead to greater toxicant exposure and it seems to reduce dependence and to support to abstinence. A third limitation was that the study was underpowered to examine abstinence difference among treatment conditions and the duration of follow-up was short. However, these preliminary results indicate that a future larger trial with longer follow-up is warranted. Finally, this study is generalizable to only one type of near nicotine-free cigarettes.

In summary, reduced nicotine content cigarettes of at least 0.05 mg nicotine yield can lead to reductions in toxicant exposure by way of changing smoking behavior and in dependence

and can possibly facilitate abstinence among smokers interested in quitting. These cigarettes can be used potentially as a cessation tool. More research should be conducted on the threshold dose for nicotine addiction during the extinction phase and factors that moderate the threshold dose, the effects of reduced nicotine content cigarettes on vulnerable populations and adjunctive methods that might facilitate cessation.

Acknowledgements

This study was funded by P50 DA013333.

References

1. Stratton, K.; Shetty, P.; Wallace, R.; Bondurant, S. Clearing the Smoke: Assessing the Science Base for Tobacco Harm Reduction. Institute of Medicine, National Academy Press; Washington, DC: 2001.
2. Royal College of Physicians. A Report by the Tobacco Advisory Group of the Royal College of Physicians. Royal College of Physicians; London: 2007. Harm reduction in nicotine addiction. Helping people who can't quit.; p. 176
3. Hatsukami, D.; Hecht, SS. Hope or hazard: what research tells us about ‘potentially reduced-exposure’ tobacco products. University of Minnesota Transdisciplinary Tobacco Use Research Center; Minneapolis: 2005. Available from http://www.rwjf.org/files/research/Hope%20or%20Hazard.pdf [9 December 2009]
4. Hatsukami D, Joseph AM, LeSage M, Jensen J, Murphy SE, Pentel PR, et al. Developing the science base for reducing tobacco harm. Nicotine Tob Res. 2007; 9:S537–53. [PubMed: 18067031]
5. US Department of Health and Human Services (USDHHS). The Health Consequences of Smoking: Nicotine and Addiction. A Report of the Surgeon General. USDHHS; Rockville, MD: 1988.
6. National Cancer Institute. Risks Associated with Smoking Cigarettes with Low Machine-Measured Yields of Tar and Nicotine. Smoking and Tobacco Control Monograph no. 13. US Department of Health and Human Services, National Institutes of Health, National Cancer Institute; Bethesda, MD: 2001.
7. Hecht SS, Murphy SE, Carmella SG, Li S, Jensen J, Le C, et al. Similar uptake of lung carcinogens by smokers of regular, light, and ultralight cigarettes. Cancer Epidemiol Biomarkers Prev. 2005; 14:693–8. [PubMed: 15767351]
8. Bernert JT, Jain RB, Pirkle JL, Wang L, Miller BB, Sampson EJ. Urinary tobacco-specific nitrosamines and 4-aminobiphenyl hemoglobin adducts measured in smokers of either regular or light cigarettes. Nicotine Tob Res. 2005; 7:729–38. [PubMed: 16191744]
9. Benowitz NL, Hall SM, Stewart S, Wilson M, Dempsey D, Jacob P 3RD. Nicotine and carcinogen exposure with smoking of progressively reduced nicotine content cigarette. Cancer Epidemiol Biomarkers Prev. 2007; 16:2479–85. [PubMed: 18006940]
10. Benowitz NL, Jacob P III, Herrera B. Nicotine intake and dose response when smoking reduced-nicotine content cigarettes. Clin Pharmacol Ther. 2006; 80:703–14. [PubMed: 17178270]
11. Hatsukami DK, Lemmonds C, Zhang Y, Murphy SE, Le C, Carmella SG, et al. Evaluation of carcinogen exposure in people who used ‘reduced exposure’ tobacco products. J Natl Cancer Inst. 2004; 96:844–52. [PubMed: 15173268]
12. Stepanov I, Jensen J, Hatsukami D, Hecht SS. Tobacco-specific nitrosamines in new tobacco products. Nicotine Tob Res. 2006; 8:309–13. [PubMed: 16766423]
13. Shiffman S, Dresler CM, Hajek P, Gilburt SJ, Targett DA, Strahs KR. Efficacy of a nicotine lozenge for smoking cessation. Arch Intern Med. 2002; 162:1267–76. [PubMed: 12038945]
14. Fiore, MC.; Bailey, W.; Cohen, S.; Dorfman, S.; Goldstein, M.; Gritz, E., et al. Treating Tobacco Use and Dependence. Clinical Practice Guideline. US Department of Health and Human Services Public Health Service; Rockville, MD: 2000.
15. Jacob, P., III; Byrd, GD. Use of chromatographic and mass spectrometric techniques for the determination of nicotine and its metabolites.. In: Gorrod, JW.; Jacob, P., III, editors. Analytical

Determination of Nicotine and Related Compounds and Their Metabolites. Elsevier; Amsterdam: 1999. p. 191-224.

16. Hecht SS, Carmella SG, Murphy SE. Effects of watercress consumption on urinary metabolites of nicotine in smokers. Cancer Epidemiol Biomarkers Prev. 1999; 8:907–13. [PubMed: 10548320]
17. Carmella SG, Han S, Fristad A, Yang Y, Hecht SS. Analysis of total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in human urine. Cancer Epidemiol Biomarkers Prev. 2003; 12:1257–61. [PubMed: 14652291]
18. Stepanov I, Hecht SS. Tobacco-specific nitrosamines and their pyridine-N-glucuronides in the urine of smokers and smokeless tobacco users. Cancer Epidemiol Biomarkers Prev. 2005; 14:885–91. [PubMed: 15824160]
19. Carmella SG, Le KA, Hecht SS. Improved method for determination of 1-hydroxypyrene in human urine. Cancer Epidemiol Biomarkers Prev. 2004; 13:1261–4. [PubMed: 15247141]
20. Carmella SG, Chen M, Zhang Y, Zhang S, Hatsukami DK, Hecht SS. Quantitation of acrolein-derived (3-hydroxypropyl)mercapturic acid in human urine by liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry: effects of cigarette smoking. Chem Res Toxicol. 2007; 20:986–90. [PubMed: 17559234]
21. Scherer G, Engl J, Urban M, Gilch G, Janket D, Riedel K. Relationship between machine-derived smoke yields and biomarkers in cigarette smokers in Germany. Regul Toxicol Pharmacol. 2007; 47:171–83. [PubMed: 17034917]
22. Hatsukami D, McBride C, Pirie P, Hellerstedt W, Lando H. Effects of nicotine gum on prevalence and severity of withdrawal in female cigarette smokers. J Subst Abuse. 1991; 3:427–40. [PubMed: 1821296]
23. Hughes JR, Gust SW, Skoog K, Keenan RM, Fenwick JW. Symptoms of tobacco withdrawal: a replication and extension. Arch Gen Psychiatry. 1991; 48:52–9. [PubMed: 1984762]
24. Hughes JR, Hatsukami D. Signs and symptoms of tobacco withdrawal. Arch Gen Psychiatry. 1986; 43:289–94. [PubMed: 3954551]
25. Hatsukami D, Anton D, Keenan R, Callies A. Smokeless tobacco abstinence effects and nicotine gum dose. Psychopharmacology. 1992; 106:60–6. [PubMed: 1738794]
26. Hatsukami D, Huber M, Callies A. Physical dependence on nicotine gum: effect of duration on use. Psychopharmacology. 1993; 111:449–56. [PubMed: 7870986]
27. Hatsukami D, Gust SW, Keenan R. Physiologic and subjective changes from smokeless tobacco withdrawal. Clin Pharmacol Ther. 1987; 41:103–7. [PubMed: 3802698]
28. Heatherton TF, Koslowski LT, Frecker RC, Fagerström K-O. The Fagerström Test for nicotine dependence: a revision of the Fagerström Tolerance Questionnaire. Br J Addict. 1991; 86:1119–27. [PubMed: 1932883]
29. Donny EC, Houtsmuller E, Stitzer ML. Smoking in the absence of nicotine: behavioral, subjective and physiological effects over 11 days [see Comment]. Addiction. 2007; 102:324–34. [PubMed: 17222288]
30. Carmella SG, Chen M, Han S, Briggs A, Jensen J, Hatsukami DK, et al. Effects of smoking cessation on eight urinary tobacco carcinogen and toxicant biomarkers. Chem Res Toxicol. 2009; 22:734–41. [PubMed: 19317515]
31. Hecht SS, Carmella SG, Chen M, Dor Koch JF, Miller AT, Murphy SE, et al. Quantitation of urinary metabolites of a tobacco-specific lung carcinogen after smoking cessation. Cancer Res. 1999; 59:590–6. [PubMed: 9973205]
32. Stepanov I, Carmella SG, Han S, Pinto A, Strasser AA, Lerman C, et al. Evidence for endogenous formation of N′-nitrosonornicotine in some long-term nicotine patch users. Nicotine Tob Res. 2009; 11:99–105. [PubMed: 19246447]
33. Rose J, Behm F. Effects of low nicotine content cigarettes on smoke intake. Nicotine Tob Res. 2004; 6:309–19. [PubMed: 15203805]
34. Hughes JR, Hatsukami DK. The nicotine withdrawal syndrome: a brief review and update. International Journal of Smoking Cessation. 1992; 1:21–6.
35. Baldinger B, Hasenfratz M, Battig K. Effects of smoking abstinence and nicotine abstinence on heart rate, activity and cigarette craving under field conditions. Hum Psychopharmacol. 1995; 10:127–36.

36. Breland AB, Buchhalter AR, Evans SE, Eissenberg T. Evaluating acute effects of potential reduced-exposure products for smokers: clinical laboratory methodology. Nicotine Tob Res. 2002; 4:S131–40. [PubMed: 12573174]
37. Buchhalter AR, Schrinel L, Eissenberg T. Withdrawal-suppressing effects of a novel smoking system: comparison with own brand, not own brand, and de-nicotinized cigarettes. Nicotine Tob Res. 2001; 3:111–8. [PubMed: 11403724]
38. Butschky MF, Bailey D, Henningfield JE, Pickworth WB. Smoking without nicotine delivery decreases withdrawal in 12-hour abstinent smokers. Pharmacol Biochem Behav. 1995; 50:91–6. [PubMed: 7700960]
39. Dallery J, Houtsmuller EJ, Pickworth WB, Stitzer ML. Effects of cigarette nicotine content and smoking pace on subsequent craving and smoking. Psychopharmacology (Berl). 2003; 165:172–80. [PubMed: 12417964]
40. Eid NC, Fant RV, Moolchan ET, Pickworth WB. Placebo cigarettes in a spaced smoking paradigm. Pharmacol Biochem Behav. 2005; 81:158–64. [PubMed: 15894074]
41. Gross J, Lee J, Stitzer ML. Nicotine-containing versus de-nicotinized cigarettes: effects on craving and withdrawal. Pharmacol Biochem Behav. 1997; 57:159–65. [PubMed: 9164567]
42. Pickworth WB, Fant RV, Nelson RA, Rohrer MS, Henningfield JE. Pharmacodynamic effects of new de-nicotinized cigarettes. Nicotine Tob Res. 1999; 1:357–64. [PubMed: 11072433]
43. Rose J, Behm FM, Westman EC, Johnson M. Dissociating nicotine and non-nicotine components of cigarette smoking. Pharmacol Biochem Behav. 2000; 67:71–81. [PubMed: 11113486]
44. Brauer L, Behm F, Lane J, Westman E, Perkins C, Rose J. Individual differences in smoking reward from de-nicotinized cigarettes. Nicotine Tob Res. 2001; 3:101–9. [PubMed: 11403723]
45. Rose J, Behm FM, Westman EC, Bates JE, Salley A. Pharmacologic and sensorimotor components of satiation in cigarette smoking. Pharmacol Biochem Behav. 2003; 76:243–50. [PubMed: 14592675]
46. Buchhalter AR, Acosta MC, Evans SE, Breland AB, Eissenberg T. Tobacco abstinence symptom suppression: the role played by the smoking-related stimuli that are delivered by denicotinized cigarettes. Addiction. 2005; 100:550–9. [PubMed: 15784070]
47. Henningfield J, Benowitz NL, Slade J, Houston TP, Davis RM, Deitchman SD. Reducing the addictiveness of cigarettes. Council on Scientific Affairs, American Medical Association. Tob Control. 1998; 7:281–93. [PubMed: 9825424]
48. Becker KM, Rose JE, Albino AP. A randomized trial of nicotine replacement therapy in combination with reduced-nicotine cigarettes for smoking cessation. Nicotine Tob Res. 2008; 10:1139–48. [PubMed: 18629723]
49. Rose J, Behm FM, Westman EC, Kukovich P. Precessation treatment with nicotine skin patch facilitates smoking cessation. Nicotine Tob Res. 2006; 8:89–101. [PubMed: 16497603]
50. Rose J, Behm F, Westman EC, Bates JE, Salley A. Pharmacologic and sensorimotor components for satiation in cigarette smoking. Pharmacol Biochem Behav. 2003; 76:243–50. [PubMed: 14592675]

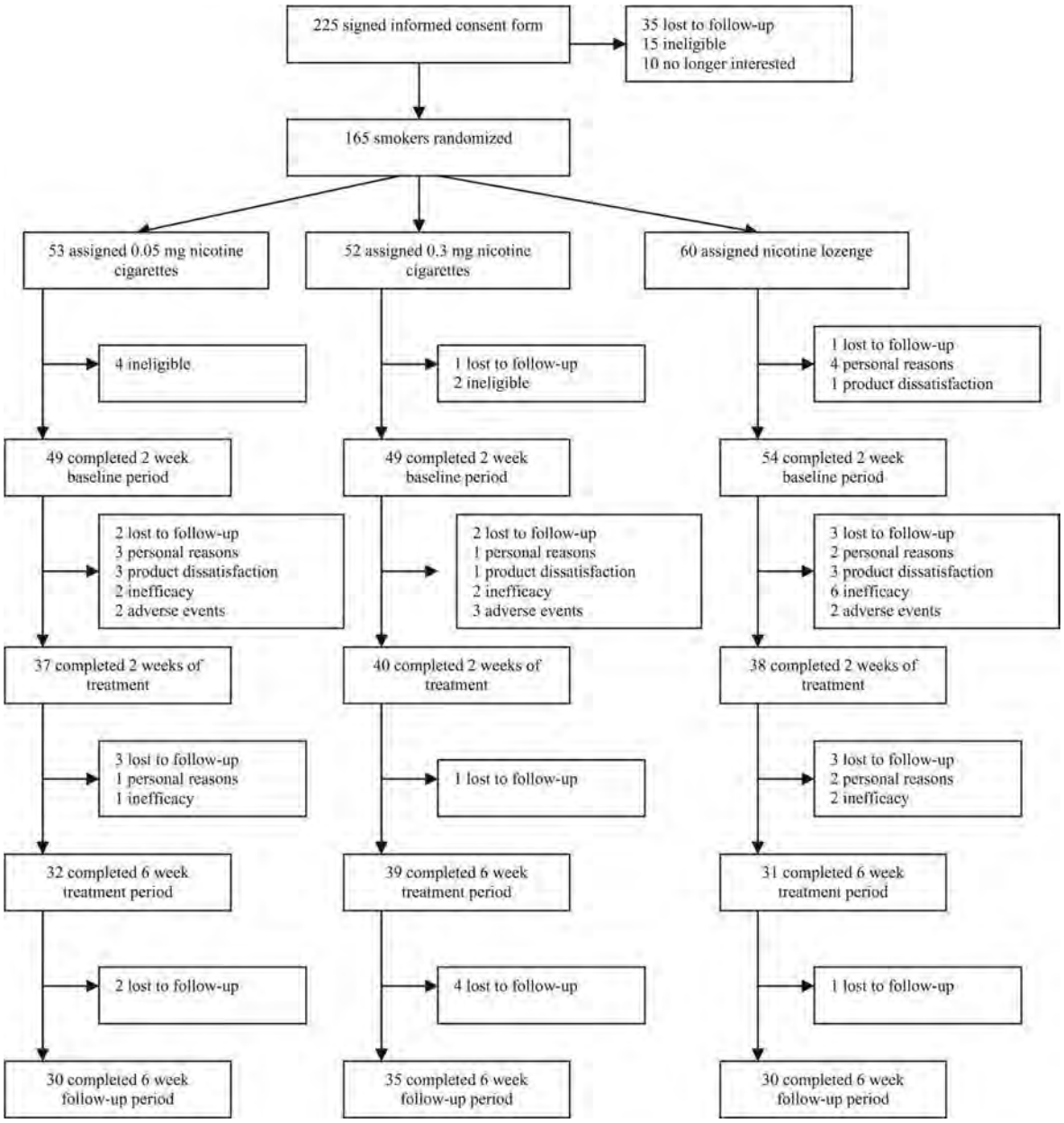

Figure 1.
Flow of subjects through study

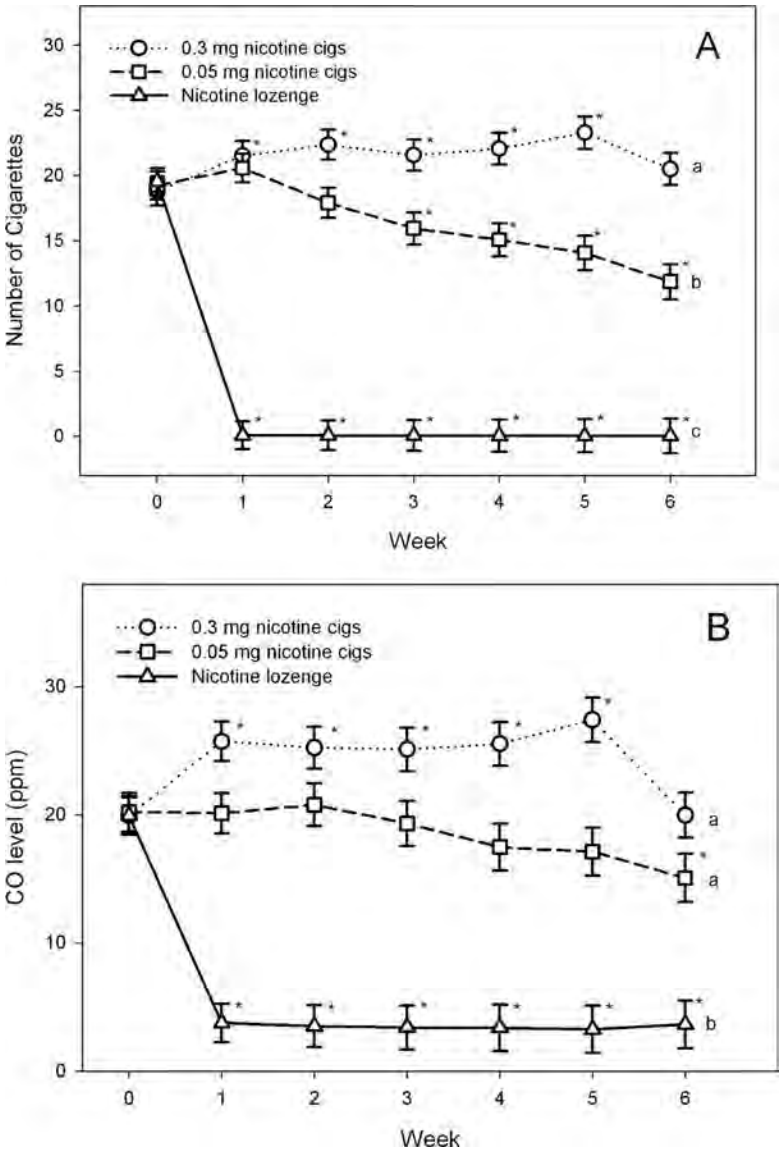

Figure 2.

Least squares (LS) mean (±standard error) of number of cigarettes smoked per day and exhaled carbon monoxide (CO). *$P < 0.05$ at that visit compared to baseline (within-group comparison). Groups with different letters were significantly different ($P < 0.05$) at the week 6 treatment visit (between-group comparison). For example, cigarettes per day are significantly different between each of the groups, but CO concentrations are significantly different between the nicotine lozenge group and each of the two cigarette groups, but the two cigarette groups are not different from each other

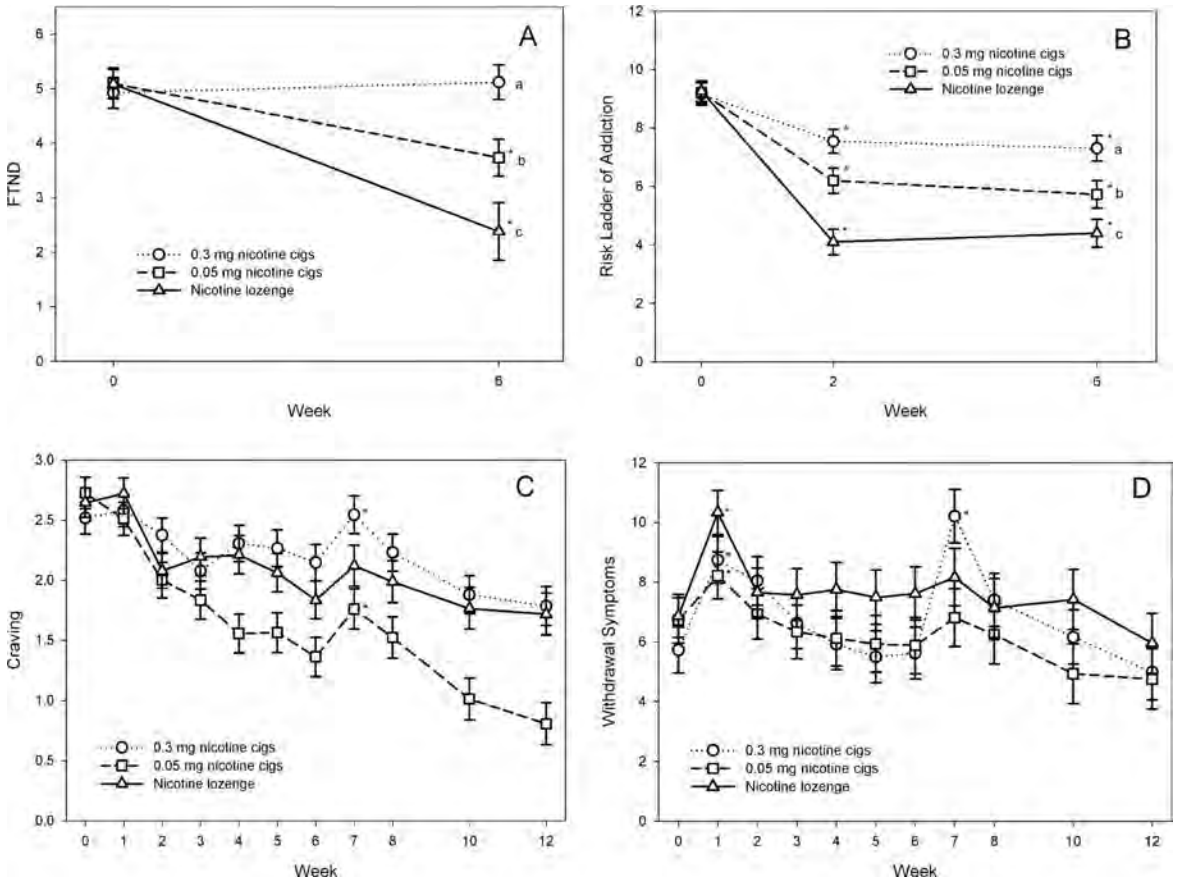

Figure 3.

Least squares (LS) mean [±standard error (SE)] of Fagerstrom Test for Nicotine Dependence (FTND) score and perceived risk for addiction score (Panels A and B). *P < 0.05 at that visit compared to baseline (within-group comparison). Groups with different letters were significantly different (P < 0.05) at the week 6 treatment visit (between-group comparison). Least squares (LS) mean (±SE) of craving and withdrawal symptoms craving and withdrawal symptoms (Panels C and D). *P < 0.05 at that visit compared to the previous visit (i.e. week 1 versus week 0 and week 7 versus week 6)

Table 1

Baseline demographics and smoking history of subjects in each of the three treatment groups. In instances where data are missing, the total number of subjects used in calculating values is fewer than the 165 randomized to treatment.

Demographics	0.3 mg nicotine cigarettes (n = 52)		0.05 mg nicotine cigarettes (n = 53)		Nicotine lozenges (n = 60)		P-value
Age (years)	51	39.4 ± 14.0	53	40.7 ± 13.3	59	43.1 ± 12.4	0.3312
Female	26	50.0%	23	43.4%	29	48.3%	0.6967
Non-Hispanic whites	47	90.4%	44	83.0%	49	81.7%	0.7653
Education							0.8782
Less than high school graduate	2	3.9%	2	3.8%	3	5.0%	
High school graduate	12	23.1%	11	20.8%	17	28.3%	
Greater than high school graduate	37	71.2%	40	75.5%	39	65.0%	
Marital status							0.1052
Never married	22	42.3%	25	47.2%	22	36.7%	
Currently married	22	42.3%	17	32.1%	16	26.7%	
Currently not married	8	15.4%	11	20.8%	21	35.0%	
Cigarettes per day	52	19.8 ± 7.8	53	21.1 ± 8.1	60	21.3 ± 9.6	0.6066
Duration of having smoked at this rate (years)	52	15.4 ± 13.0	53	14.1 ± 12.6	59	15.7 ± 14.1	0.7960
Age smoking first cigarette (years)	52	14.4 ± 2.9	53	15.5 ± 4.8	60	14.5 ± 2.9	0.2518
Age becoming a regular smoker (years)	52	16.5 ± 3.0	52	19.4 ± 7.3	58	17.7 ± 4.1	0.0195
Motivation to quit (0–10 scale)	52	9.0 ± 1.1	53	9.2 ± 1.0	59	9.2 ± 1.3	0.6928
Number of quit attempts							0.4482
1–2	13	25.0%	17	32.1%	13	21.7%	
3–5	14	26.9%	14	26.4%	24	40.0%	
6–10	12	23.1%	10	18.9%	13	21.7%	
11+	10	19.2%	4	7.6%	9	15.0%	
Spouse or significant other smokes	20	38.5%	20	37.7%	21	35.0%	0.9515

Table 2

Geometric means of biomarkers at baseline and weeks 2 and 6 of treatment period by treatment groups. Values are for all subjects from whom data were collected at the visit in question.

	Geometric mean (95% confidence interval)		
Biomarkers	**Baseline**	**Week 2**	**Week 6**
Total cotinine[1]			
0.3 mg cigarettes	4057 (3323, 4952)	2150 (1696, 2725)*	2093 (1611, 2719)*a
0.05 mg cigarettes	4216 (3492, 5090)	278 (174, 442)*	188 (111, 319)*b
Nicotine lozenge	3917 (3399, 4514)	2291 (1708, 3073)*	2154 (1312, 3536)*a
Total NNAL[2]			
0.3 mg cigarettes	0.96 (0.73, 1.26)	0.54 (0.41, 0.69)*	0.47 (0.30, 0.73)*a
0.05 mg cigarettes	0.92 (0.70, 1.21)	0.34 (0.20, 0.57)*	0.20 (0.11, 0.34)*b
Nicotine lozenge	1.06 (0.84, 1.35)	0.24 (0.18, 0.32)*	0.14 (0.07, 0.26)*b
Total NNN[2]			
0.3 mg cigarettes	0.10 (0.06, 0.16)	0.09 (0.06, 0.14)	0.06 (0.04, 0.10)a
0.05 mg cigarettes	0.09 (0.05, 0.15)	0.06 (0.04, 0.11)	0.03 (0.02, 0.07)*ab
Nicotine lozenge	0.08 (0.05, 0.12)	0.02 (0.01, 0.04)*	0.02 (0.01, 0.04)*b
Total 1-HOP[2]			
0.3 mg cigarettes	0.84 (0.70, 1.02)	0.95 (0.58, 1.53)	0.73 (0.59, 0.90)a
0.05 mg cigarettes	0.89 (0.71, 1.12)	0.75 (0.56, 1.01)	0.57 (0.42, 0.78)*a
Nicotine lozenge	0.94 (0.71, 1.24)	0.40 (0.29, 0.56)*	0.34 (0.21, 0.57)*b
3-HPMA[2]			
0.3 mg cigarettes	3662 (2868, 4674)	2838 (2226, 3619)	2732 (2110, 3537)a
0.05 mg cigarettes	3320 (2667, 4134)	1639 (1215, 2211)*	1453 (1039, 2032)*b
Nicotine lozenge	3445 (2539, 4673)	911 (670, 1239)*	1062 (749, 1508)*b
S-PMA[2]			
0.3 mg cigarettes	2.21 (1.54, 3.18)	1.30 (0.88, 1.92)*	1.35 (0.94, 1.93)*a
0.05 mg cigarettes	2.46 (1.68, 3.62)	1.54 (1.03, 2.31)*	0.76 (0.48, 1.20)*b
Nicotine lozenge	2.69 (1.95, 3.72)	0.33 (0.22, 0.49)*	0.48 (0.30, 0.78)*b

Groups with different letters were significantly different ($P < 0.05$) at the week 6 treatment visit (between-group comparison). For example, total cotinine is significantly different between the 0.05 mg cigarette group with 0.3 mg cigarette group and with nicotine lozenge group, but the 0.3 mg cigarette group is not significantly different from the nicotine lozenge group. Total NNAL: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides; total NNN: *N′*-nitrosonornicotine and its glucuronide; total 1-HOP: 1-hydroxypyrene and its glucuronide and sulfate; 3-HPMA: 3-hydroxypropylmercapturic acid; S-PMA: *S*-phenylmercapturic acid.

[1] ng/ml.

[2] pmol/mg creatinine.

*$P < 0.05$ at that visit compared to baseline (within-group comparison).

Table 3

Continuous (past 4 weeks) and point-prevalence (past 1 week) post-treatment abstinence rates. Products with different superscript letters were significantly different ($P < 0.05$).

	Treatments						
	0.3 mg nicotine cigarettes (n = 52)		Nicotine lozenges (n = 60)		0.05 mg nicotine cigarettes (n = 53)		
	# abstinent	%	# abstinent	%	# abstinent	%	P-value
Continuous abstinence							
[1]CO verified	11	21.2	21	35.0	23	43.4	0.0508
[2]CO and cotinine verified	7	13.5	11	18.3	16	30.2	0.0913
CO verified point prevalence abstinence							
Follow-up week							
1	18	34.6	25	41.7	22	41.5	0.6954
2	17	32.7	25	41.7	29	54.7	0.0719
4	12	23.1	22	36.7	23	43.4	0.0829
6	12	23.1[a]	22	36.7[a,b]	25	47.2[b]	0.0357
CO and cotinine verified point prevalence abstinence							
Follow-up week							
2	12	23.1[a]	16	26.7[a]	24	45.3[b]	0.0298
4	8	15.4[a]	13	21.7[a]	21	39.6[b]	0.0120
6	7	13.5[a]	12	20.0[a,b]	19	35.9[b]	0.0192

[1]Carbon monoxide (CO) verified abstinence represents abstinence from cigarettes but usage of nicotine lozenge is allowed.

[2]CO and cotinine verified represents abstinence from all nicotine-containing products, including nicotine lozenge.

HHS Public Access

Author manuscript

Tob Control. Author manuscript; available in PMC 2016 November 01.

Published in final edited form as:

Tob Control. 2015 November ; 24(6): 536–539. doi:10.1136/tobaccocontrol-2014-051797.

Greater reductions in nicotine exposure while smoking very low nicotine content cigarettes predict smoking cessation

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Abstract

Objective—Reducing the nicotine content of cigarettes is a potential regulatory strategy that may enable cessation. The present study investigated the effect of nicotine exposure while smoking very low nicotine content (VLNC) cigarettes on cessation outcomes. The roles of possible sources of nicotine were also explored, including the VLNC cigarette and co-use of cigarettes with normal nicotine content.

Methods—A secondary data analysis of two analogous randomized trials of treatment-seeking, adult daily smokers (n=112) who were instructed to smoke VLNC cigarettes for 6 weeks and then make a quit attempt. Controlling for baseline demographic and smoking features, the association between reductions in nicotine exposure during the 6 week trial, assessed by urinary total cotinine, and biomarker-confirmed smoking abstinence one month later was tested. Subsequent analyses controlled for the effects of the frequency of VLNC and normal nicotine content cigarette use, and the nicotine yield of the VLNC cigarette (0.05 mg vs 0.09 mg).

Results—Greater reductions in nicotine exposure while smoking VLNC cigarettes predicted abstinence independent of individual differences in baseline smoking, cotinine, dependence, gender and study. Nicotine reduction was largest among individuals who were assigned to smoke a VLNC cigarette with lower nicotine yield, and who smoked fewer normal nicotine content and VLNC cigarettes.

Conclusions—In the context of nicotine regulations and corresponding research, factors that undermine nicotine reduction must be addressed, including the availability and use of cigarettes with normal nicotine content, and not sufficiently reducing the nicotine yield of cigarettes. Maximizing nicotine reduction may facilitate smoking cessation.

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Contributors SSD and ECD conceived the idea for this study. DKH conceived the idea for the original research studies, and LAH collected the data and assisted in the original study designs. SSD and ECD contributed to the data analysis and interpretations, writing and revisions of the manuscript. DKH also assisted in the interpretation of study findings. DKH and LAH provided feedback for drafts of the manuscript.

Data sharing Readers are encouraged to contact the authors for additional information about the data.

Competing interests None declared.

Keywords

smoking cessation; cotinine; nicotine reduction; tobacco control; very low nicotine content cigarette

According to the World Health Organization (WHO) Framework Convention on Tobacco Control (Article 9), guidelines may be developed to regulate the content and emissions of tobacco products [1]. As nicotine in tobacco sustains smoking [2], reducing the nicotine content in cigarettes could improve public health by increasing cessation rates [3, 4].

The potential impact of nicotine regulations on smoking has been evaluated using very low nicotine content (VLNC) cigarettes. VLNC cigarettes contain much less nicotine in the tobacco and, when smoked, yield substantially less nicotine (<0.1 mg) [3] than conventional cigarettes (e.g., 0.8 mg)[5-7]. Within a week of switching to VLNC cigarettes, smokers have markedly reduced levels of nicotine metabolites that are similar to abstinent smokers [8, 9] and remain low with continued use [10, 11].

Reduced nicotine exposure from VLNC cigarettes, however, have not consistently facilitated cessation. Among treatment-seeking smokers, 6 weeks of VLNC cigarettes use alone or with nicotine replacement therapy increased cessation in some [11-13] but not all investigations [10]. Specifically, after smoking VLNC cigarettes alone, only 24.1% of participants were abstinent, compared to 35.9% in an analogous study [11]. Understanding what processes enable smoking cessation is critical to explain any underestimated effects of VLNC cigarettes on abstinence, and to determine the impact of nicotine reduction as a regulatory strategy. To this end, the present study examined if lower nicotine exposure improved cessation rates when smoking VLNC cigarettes. Factors that may undermine nicotine reduction efforts and corresponding abstinence rates were also explored, including co-use of conventional cigarettes, the nicotine yield of VLNC cigarettes, and number of VLNC cigarettes smoked.

Methods

Participants

Treatment-seeking, adult daily smokers were recruited from the community via advertisement as part of two larger studies (N_{2010}=165; N_{2013}=235) comparing the effect of VLNC cigarettes (0.05–0.09 mg nicotine yield) on smoking outcomes to other nicotine-containing products (e.g., 0.3 mg cigarettes, lozenge, patch)[10, 11]. Eligible participants smoked 10-40 cigarettes per day (CPD). Exclusion criteria included pregnancy/nursing, unstable physical/psychiatric conditions, contraindications for medicinal nicotine use, and recent other tobacco/nicotine product use. The University of Minnesota Institutional Review Board approved the studies.

The present study examined individuals assigned to use VLNC cigarettes only and used the products for at least one week (n=112). Participants were generally Caucasian (83.9%) and middle-aged (mean=44.75 years, standard deviation (SD)=12.88), with equally represented genders (47.3% male). Original publications provide additional details [10, 11].

Procedure

Participants experienced nearly identical protocols, except most individuals in the later study (76%) smoked 0.09 mg nicotine yield cigarettes because the original 0.05 mg cigarettes were unavailable. After smoking usual brand cigarettes for a 2-week baseline period, participants were instructed to smoke the VLNC cigarette exclusively for 6 weeks. Participants were provided with 150% of their baseline CPD and reported cigarette use using a daily diary. They were encouraged to report (and not penalized for) non-study cigarette use. A weekly, brief standardized counseling session prepared participants for a quit attempt at the end of the study. Both studies evaluated biomarker-confirmed abstinence at week 12 (6-weeks post-quit attempt).

Measures

Nicotine exposure at baseline, week 6, and week 12 was assessed by total urinary cotinine (urinary free cotinine plus cotinine *N*-glucuronide)[14]. Change in cotinine was examined in two ways: Week 6 cotinine level controlling for baseline cotinine level and percent change in cotinine level from baseline to week 6. The cotinine outcomes were natural log transformed due to positive skew[1].

Abstinence at week 12 was defined as no VLNC or non-study cigarettes smoked during the past 7 days and carbon monoxide (CO) < 6 ng/ml. The analyses were replicated using cotinine (<35 ng/ml) to confirm self-reported abstinence. These analyses are omitted for brevity because they yielded similar findings and identical conclusions.

Analyses

Using logistic regression (Mplus 7.11), the association between change in cotinine levels and week 12 biomarker-confirmed abstinence from cigarettes was examined. Missing data at week 6 were handled using maximum likelihood estimation with Monte Carlo data generation. Individuals lost to follow-up after week 1 were coded as smoking at week 12. A second set of analyses accounted for sources of nicotine at week 6. Specifically, VLNC cigarette type (0.05 vs 0.09 mg), week 6 non-study and VLNC CPD, and study non-compliance (i.e., any self-reported non-study nicotine\tobacco use after week 2) were added as predictors of both change in cotinine and week 12 abstinence. Other tobacco product use was not considered due to infrequent use (n=2). Covariates included baseline cotinine, CPD, and Fagerström Test for Nicotine Dependence score excluding CPD item (FTND), gender, and study (2010 as reference category)[15].

Results

At baseline, participants smoked 20.09 CPD (SD=1.76) and were moderately nicotine dependent (FTND=4.46, SD=1.76). The retention rate was 74% for Week 6 and 63% for Week 12. Table 1 summarizes observed relations between cotinine levels and study outcomes.

[1]Percent change was transformed (absolute value of percent change minus 101) in order to conduct the natural log transformation on a distribution with a positive skew with values greater than or equal to 1.

Lower urinary total cotinine level after smoking VLNC cigarettes for 6 weeks increased the odds of cessation 6 weeks later (Odds Ratio (OR)=0.52, 95% CI:0.34-0.80, *p*=.003). This effect was replicated with percent change in cotinine (OR=0.46, 95% CI:0.27-0.79, *p*=.005)[23]. Effects were not moderated by study or gender (i.e., non-significant interaction terms).

Controlling for other covariates and sources of nicotine exposure, Week 6 urinary total cotinine level was significantly higher in the 2010 study (standardized:β=−0.29, *p*=.046), and among individuals who smoked 0.09 mg VLNC cigarettes (β=0.31, *p*=.03), reported more non-study (β=0.34, *p*=.001) and VLNC (β=.26, *p*=.01) CPD at Week 6, and marginally associated with non-compliance (β=0.18, *p*=.09). Week 6 urinary total cotinine continued to predict abstinence (OR=0.44, 95% CI:0.22-0.86, *p*=.03), after controlling for sources of nicotine exposure. Percent change in cotinine was significantly associated with non-study (β=0.43, *p*<.001) and VLNC CPD at Week 6 (β=0.22, *p*=.045). Percent change in cotinine remained significantly related to abstinence (OR=0.39, 95% CI:0.18-0.86, *p*=.007), after controlling for sources of nicotine.

Conclusions

Greater reductions in nicotine exposure when smoking VLNC cigarettes were associated with increased cigarette abstinence. This is consistent with previous research [11-13] and literature reviews [16, 17] indicating that reducing the nicotine content of cigarettes may improve public health.

The extent of nicotine reduction was affected by the co-use of conventional cigarettes with normal nicotine content. Individuals who smoked conventional cigarettes exhibited higher nicotine exposure, which corresponded with difficulty quitting smoking (6.3% quit relative to 51.2% who reported only using VLNC cigarettes). Smoking conventional cigarettes likely maintained the reinforcing properties of cigarettes and nicotine dependence, undermining the effect of VLNC cigarettes on cessation. Thus, research conducted in an open marketplace with widespread availability of conventional cigarettes may underestimate the impact of nicotine reduction due to non-compliance. Research should utilize methods to reduce non-compliance (e.g., incentivizing compliance, limit access to conventional cigarettes) and report how non-compliance impacts study findings.

Similarly, characteristics of VLNC cigarette use, such as its nicotine yield and number smoked per day, increased nicotine exposure. While the nicotine yield of the VLNC cigarettes were substantially reduced relative to conventional cigarettes (0.8 mg), almost doubling their nicotine yield (from 0.05 to 0.09 mg) along with an increased smoking rate could sustain nicotine exposure at a level that impedes cessation. This may partially explain the differential outcomes in previous research [10, 11]. Thus, to facilitate cessation in both a research and regulatory context, it is imperative to sufficiently lower the nicotine yield of cigarettes.

[2]Analyses were replicated after excluding participants who dropped out of the study prior to week 6. The pattern of findings was successfully replicated for both outcomes.
[3]Analyses with percent change excluded three outlier week 6 cotinine cases (at least 3 standard deviations from the mean).

Several study limitations should be acknowledged. The sample of completers was relatively small and not nationally representative, which limits generalizability of findings. Non-completers were assumed to be smoking, which may have underestimated cessation rates. Non-compliance was self-reported. Thus, associations between non-compliance and cessation may have been underestimated, leading to a continued association between cotinine and cessation. As a post-hoc secondary analysis that did not experimentally manipulate nicotine exposure was conducted, associations may be partly explained by unmeasured individual differences in compliance, motivation, or environment (e.g., spousal smoking, smoke-free policies) that also affect cessation. This issue was partly addressed by controlling for baseline characteristics associated with cessation (e.g., FTND, CPD, cotinine). Furthermore, the nicotine yield of the assigned VLNC cigarette predicted abstinence rates, reinforcing the importance of nicotine in quit rates.

Finally, additional research is needed to determine how nicotine exposure from other tobacco products would impact cessation. To date, many investigations of VLNC cigarettes, including this study, have excluded individuals who regularly use other tobacco or nicotine products. With the evolving marketplace of non-combustible products, including the increasing popularity of e-cigarettes, it is critical to determine how VLNC cigarettes may be more or less effective in this real-life context. While the present study suggests that nicotine from conventional cigarettes may undermine cessation while smoking VLNC cigarettes, it is unlikely that this effect will generalize to all nicotine and tobacco products. For instance, research suggests that nicotine exposure from the nicotine patch when used alongside VLNC cigarettes leads to lower rates of smoking [10, 18], which may *facilitate* quit attempts. As such, to inform regulatory decisions, it is imperative to determine which alternative sources of nicotine affect cessation when using VLNC cigarettes and in what direction (i.e., facilitate vs impede). It is suspected that several factors may come into play, particularly factors that would affect the reinforcing properties of smoking such as the extent to which the product resembles a cigarette with regards to its sensory aspects and nicotine delivery, and patterns of co-use (e.g., simultaneous use as opposed to same-day).

In summary, studies examining the impact of reduced nicotine content cigarettes on cessation may be affected by the availability of non-regulated cigarettes and other nicotine or tobacco products. Furthermore, enacting a nicotine standard that does not sufficiently reduce the nicotine content of cigarettes may impede cessation efforts. Of note, poorer cessation outcomes were seen with a relatively small increase in nicotine yield of VLNC cigarettes (from 0.05 mg to 0.09 mg). Thus, to maximize public health benefits, it is critical to reduce the nicotine yield of all cigarettes to the lowest possible level while encouraging reduced smoking.

Acknowledgements

The authors thank Dr. Neal Benowitz for critically reviewing an initial draft of the manuscript.

Funding Funding for this study was provided by National Institutes of Health (NIH) grants R01 DA025598 (PI: Dr. Dorothy Hatsukami) and F31AA0022291 (PI: Sarah Dermody). The NIH had no role in the study design, data collection or analysis, interpretation of data or writing of the report, or the decision to submit the paper for publication.

References

1. World Health Organization. WHO framework convention on tobacco control. WHO; Geneva: 2003.
2. US Department of Health Human Services. Nicotine addiction: A report of the surgeon general. USDHSS; Rockville, MD: 1988.
3. Benowitz NL, Henningfield JE. Establishing a nicotine threshold for addiction. The implications for tobacco regulation. N Engl J Med. 1994; 331:123. [PubMed: 7818638]
4. Hatsukami DK, Perkins KA, LeSage MG, et al. Nicotine reduction revisited: science and future directions. Tob Control. 2010; 19:e1–10. [PubMed: 20876072]
5. Kozlowski LT, Mehta NY, Sweeney CT, et al. Filter ventilation and nicotine content of tobacco in cigarettes from Canada, the United Kingdom, and the United States. Tob Control. 1998; 7:369–375. [PubMed: 10093170]
6. Benowitz NL, Jacob P, Herrera B. Nicotine intake and dose response when smoking reduced-nicotine content cigarettes. Clin Pharmacol Ther. 2006; 80:703–714. [PubMed: 17178270]
7. Pickworth WB, Nelson RA, Rohrer MS, et al. Pharmacodynamic effects of new de nicotinized cigarettes. Nicotine Tob Res. 1999; 1:357–364. [PubMed: 11072433]
8. Hatsukami DK, Heishman SJ, Vogel RI, et al. Dose–response effects of spectrum research cigarettes. Nicotine Tob Res. 2013; 15:1113–1121. [PubMed: 23178320]
9. Buchhalter AR, Acosta MC, Evans SE, et al. Tobacco abstinence symptom suppression: the role played by the smoking-related stimuli that are delivered by denicotinized cigarettes. Addiction. 2005; 100:550–559. [PubMed: 15784070]
10. Hatsukami DK, Hertsgaard LA, Vogel RI, et al. Reduced nicotine content cigarettes and nicotine patch. Cancer Epidemiol Biomarkers Prev. 2013; 22:1015–1024. [PubMed: 23603206]
11. Hatsukami DK, Kotlyar M, Hertsgaard LA, et al. Reduced nicotine content cigarettes: effects on toxicant exposure, dependence and cessation. Addiction. 2010; 105:343–355. [PubMed: 20078491]
12. Becker KM, Rose JE, Albino AP. A randomized trial of nicotine replacement therapy in combination with reduced-nicotine cigarettes for smoking cessation. Nicotine Tob Res. 2008; 10:1139–1148. [PubMed: 18629723]
13. Walker N, Howe C, Bullen C, et al. The combined effect of very low nicotine content cigarettes, used as an adjunct to usual Quitline care (nicotine replacement therapy and behavioural support), on smoking cessation: a randomized controlled trial. Addiction. 2012; 107:1857–1867. [PubMed: 22594651]
14. Jacob, P.; Byrd, GD. Use of chromatographic and mass spectrometric techniques for the determination of nicotine and its metabolites.. In: Gorrod, JW.; Jacob, P., editors. Analytical Determination of Nicotine and Related Compounds and Their Metabolites. Elsevier; Amsterdam: 1999. p. 191-224.
15. Heatherton TF, Kozlowski LT, Frecker RC, et al. The Fagerström test for nicotine dependence: a revision of the Fagerstrom Tolerance Questionnaire. Br J Addict. 1991; 86:1119–1127. [PubMed: 1932883]
16. Donny EC, Hatsukami DK, Benowitz NL, et al. Reduced nicotine product standards for combustible tobacco products: Building an empirical basis for effective regulation. Prev Med. In Press.
17. Hatsukami DK, Benowitz NL, Donny E, et al. Nicotine reduction: Strategic research plan. Nicotince Tob Res. 2013:1003–1013.
18. Donny EC, Jones M. Prolonged exposure to denicotinized ciarettes with or without transdermal nicotine. Drug and Alcohol Dependence. 2009:23–33. [PubMed: 19446968]

What this study adds

➢ This is the first study to demonstrate that, when smoking VLNC cigarettes, lower levels of nicotine exposure prior to a quit attempt enables cessation.

➢ Smoking high nicotine content cigarettes alongside VLNC cigarettes appeared to undermine nicotine reduction efforts, and in turn, reduce quit rates. Thus, the widespread availability of high nicotine content cigarettes may lead researchers to underestimate the public health impact of a nicotine reduction strategy due to non-compliance.

➢ Relatively small differences in the nicotine yield of VLNC cigarettes (0.05 mg vs 0.09 mg yield) appeared to impact cessation rates.

Table 1

Key sample characteristics (n = 83) at each observed week 6 cotinine level

	Natural Log of Week 6 Cotinine									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Sample size	2	0	1	2	16	21	24	6	5	6
% in 2013 study	100		100	0	56.3	47.6	75.0	83.3	100	66.7
% smoking 0.09 mg yield	0		100	0	25.0	33.3	70.8	66.7	60.0	66.7
study CPD (Week 6)	0		2.6	7.9	8.7	16.9	17.8	17.2	11.4	18.0
non-study CPD (Week 6)	0		0	0	0	0	0.2	0.7	4.2	6.7
% non-compliant	0		100	0	12.5	14.3	33.3	50.0	80.0	66.7
% abstinent (Week 12)	100		100	100	81.3	57.1	47.1	16.7	20	16.7

Note. Participants who completed the Week 6 visit (n = 83) were divided into 10 groups based on their natural log of Week 6 cotinine levels (binned by rounding to the nearest whole number). Each column in the table provides descriptive statistics (means or proportions) of study outcomes for individuals who had the specified cotinine level (refer to sample size for n). The data provide qualitative support for factors that may explain the linear relation between cotinine level and quit rates. Of note, only 2 participants stopped smoking the VLNC cigarettes before Week 6 because they had quit smoking all cigarettes. Furthermore, the number of VLNC cigarettes appeared to differentiate individuals at cotinine level 4 from 5; whereas, non-compliance and higher nicotine yield of study cigarette (0.05 mg vs 0.09 mg) appeared to differentiate individuals at cotinine level 5 from 6.

Nicotine & Tobacco Research, Volume 15, Number 6 (June 2013) *1113–1121*

ORIGINAL INVESTIGATION

Dose–Response Effects of Spectrum Research Cigarettes

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Received July 21, 2012; accepted October 10, 2012

ABSTRACT

Introduction: Experimental cigarettes are needed to conduct studies examining the effects of varying doses of nicotine content on smoking behavior. The National Institute on Drug Abuse contracted with Research Triangle Institute to make such cigarettes available to researchers. The goal of this study was to determine whether cigarettes that vary in nicotine content produce an expected dose–response effect.

Method: Two studies were conducted. The first study recruited subjects from 3 sites and consisted of a single, within-subject laboratory session. Subjects first smoked 4 puffs on their usual-brand cigarette and then in double-blind, random-order, smoked 4 puffs on each experimental cigarette that contained either low nicotine (LN, 0.4 mg/g), intermediate nicotine (IN, 5.7–5.8 mg/g), or high nicotine (HN, 11.4–12.8 mg/g). Each puffing bout was separated by a 30-min interval. Subjects completed questionnaires and were assessed for vital signs after each cigarette. The second study involved 1 site and used a between-subject design in which subjects were assigned to 1 of the 3 experimental cigarettes for 1 week. Subjective responses and biomarkers of exposure were assessed.

Results: In the first study, significant dose–response effects were observed, particularly between the LN and HN cigarettes. The second study showed decreases in cigarette smoking and exposure biomarkers predominantly in the LN group, with no changes in the HN cigarette group.

Conclusions: These results are similar to those observed in prior literature, confirming that these experimental cigarettes can be used safely and with the expected pharmacological effects.

INTRODUCTION

Smoking remains a leading cause of preventable disease and premature death worldwide. Approximately one in five death is associated with cigarette smoking, and roughly half of all daily smokers will die prematurely from tobacco-related illness (Doll, Peto, Boreham, & Sutherland, 2004; Peto, Lopez, Boreham, Thun, & Heath, 1992). Nicotine is the primary addictive agent in tobacco products (U.S. Department of Health and Human Services, 1988, 2010), yet a comprehensive body of scientific literature examining the effects of nicotine reduction in cigarettes or other tobacco products does not exist (Hatsukami, Perkins et al., 2010). The availability of cigarettes with varying levels of nicotine but otherwise similar characteristics provides the opportunity to improve understanding of how nicotine and other aspects of smoking contribute to the addictive properties of cigarettes.

The availability of cigarettes varying in nicotine content is also important to scientifically determine if reducing nicotine content in cigarettes may be a viable national policy strategy. Reducing the nicotine in cigarettes to the point that they are rendered nonaddictive has the potential to significantly reduce tobacco-related mortality and morbidity by decreasing the initiation of smoking and promoting cessation (Benowitz & Henningfield, 1994; Gray et al., 2005; Zeller, Hatsukami, & Strategic Dialogue on Tobacco Harm Reduction Group, 2009). The Family Smoking Prevention and Tobacco Control Act (FSPTCA) enables the Food and Drug Administration to establish tobacco product standards, including placing limits on the allowable nicotine content of cigarettes without reducing levels to zero. Similarly, Article 9 in the Framework Convention on Tobacco Control describes the regulation of content and emissions of tobacco products.

Currently, no reduced nicotine cigarettes are available to researchers that would allow examining the effects of varying doses of nicotine on smoking behavior. To meet this need, the National Institute on Drug Abuse (NIDA) contracted with Research Triangle Institute (RTI) to assist in the development of cigarettes varying in nicotine content. At least 9 million

doi:10.1093/ntr/nts247
Advance Access publication November 22, 2012

cigarettes will be made available to the research community. After early batches were produced, the subjective effects, cardiovascular effects, and levels of nicotine and smoke exposure were determined in two pilot studies. In the first study, smokers were asked to take four puffs on their usual-brand cigarette and experimental cigarettes with three different nicotine content levels. In the second study, subjects were randomly assigned to one of three different nicotine content cigarettes for 1 week.

STUDY 1

Methods

Subjects

Subjects were recruited at three different sites, the University of Minnesota, University of Pittsburgh and the NIDA Intramural Research Program. Subjects who had previously enrolled in a smoking study or who responded to an advertisement for a smoking study were screened over the telephone to determine if they met the following eligibility criteria: 18–64 years of age, smoke at least 10 cigarettes/day with a CO > 10 parts per million (ppm) at screening, inhale when they smoked, use other forms of tobacco less than 10 days in the last 30 days, no plans to reduce or quit smoking, no use of nicotine replacement therapy, bupropion, or varenicline in the past 3 months, in good mental and physical health, not be pregnant, and not taking certain prescription medications or illicit drugs more than twice per week for the last month.

Cigarettes

Both menthol and nonmenthol cigarettes were manufactured by 22nd century (named Spectrum) under a NIDA contract with RTI. The nicotine content of the menthol and nonmenthol cigarettes as assessed by RTI were about 0.4 mg/g, 5.7–5.8 mg/g, and 11.4–12.8, mg/g, respectively (variation between menthol and nonmenthol cigarettes). The nicotine yield of these cigarettes (menthol and nonmenthol) as measured by the International Organization for Standardization method were <0.04 mg nicotine (low nicotine, LN), 0.3 mg nicotine (intermediate nicotine, IN), and 0.6 mg nicotine (high nicotine, HN) per cigarette. Tar yields, determined by Arista, were approximately 8.1–8.4, 8.6, and 9.6–9.8 mg, respectively. Menthol content, determined by RTI, were 1.23, 1.23, and 1.08 mg/g, respectively, for menthol cigarettes (yields were 0.47, 0.35, and 0.33 mg/cigarette, respectively) and nondetectable for nonmenthol cigarettes. Cigarettes did not vary in ventilation; instead, nicotine yields were achieved by blending tobacco with different nicotine content.

Study Design

Subjects attended one laboratory session during which informed consent was obtained and eligibility further assessed. Subjects first smoked their usual brand of cigarettes and then were asked to smoke in double-blind, random-order and sequential manner each of the three experimental cigarettes; each cigarette type was separated by 30-min intervals. Menthol and nonmenthol cigarettes smokers were assigned their respective preference. Subjects took four puffs (1 bout) on each cigarette type at 30-s interpuff intervals. Heart rate and blood pressure were measured immediately after each puffing bout. Alveolar carbon monoxide (CO) level was measured 2 min after each bout. Subjects then completed subjective rating scales, including the modified Cigarette Evaluation Questionnaire (mCEQ; Westman, Levin, & Rose, 1992) scored on an visual analog 0–100 scale (not at all to extremely) instead of a 1–7 scale, the Multiple Choice Procedure (MCP; Jacobs & Bickel, 1999), and the Perceived Health Risk (PHR) scale (measures perceived risk of experiencing a specific disease as a result of smoking experimental cigarettes; rated from very low risk of disease to very high risk of disease). Additional measures included assessment of characteristics of the tobacco product (e.g., flavorful, strength, harshness, amount of nicotine, like and dislike of cigarettes rated on a 0–100 scale, not at all to extremely). After the last cigarette, subjects were asked to rank the cigarettes in terms of their overall preference, including their usual brand.

Statistical Methods

Demographic and smoking history data were summarized by study site and menthol status. The mCEQ was scored as 5 subscales: Satisfaction, Psychological Reward, Aversion, Enjoyment of Respiratory Tract Sensations, and Craving Reduction (Cappelleri et al., 2007). All continuous outcomes were analyzed using a mixed effects analysis of variance model with fixed effects for baseline response (relating to their usual brand), experimental cigarette nicotine content, experimental cigarette smoking ordering, gender, study site, menthol status, interactions between nicotine content and menthol status and gender, and a random effect for subject. Rank data were analyzed using a proportional odds model with a cumulative logit link, with fixed effects for experimental cigarette nicotine content, experimental cigarette smoking ordering, gender, study site, menthol status, interactions between nicotine content and menthol status and gender, and a random effect for subject to account for the repeated nature of the data. Least-squares (LS) means ± standard errors (*SE*) were reported for each nicotine level unless otherwise noted and *p* values were adjusted for multiple comparisons using a Bonferroni correction. All significance levels were set at .05.

Results

Demographic and smoking history information of subjects recruited at the University of Minnesota (n = 20), University of Pittsburgh (n = 19), and NIDA (n = 12) are the following: mean age 39.6 years (SD = 12.6); 39.2% female; 58.8% White, 37.3% Black, and 3.9% other; 47.1% menthol smokers; mean cigarettes/day 18.6 (SD = 7.3); mean years daily smoking 19.9 years (SD = 11.9). No significant differences were observed across the sites. Information from two subjects at the University of Pittsburgh was lost for their first cigarette due to a computer malfunction. All others have complete data.

Modified Cigarette Evaluation Questionnaire

Table 1 shows the results for the mCEQ. For the subscales related to Satisfaction, Psychological Reward, Enjoyment of Respiratory Tract Sensation, and Craving Reduction, the smokers scored the HN and/or IN cigarettes significantly higher than the LN cigarettes. IN and HN cigarette scores were not significantly different on these scales. Nonmenthol compared with menthol smokers found their experimental cigarettes significantly more satisfying (59.1 ± 3.5 vs. 42.4 ± 3.8; F = 11.66, p = .001), more psychologically rewarding (43.5 ± 3.1 vs. 35.1 ± 2.9; F = 5.39, p = .022), more pleasing to the respiratory tract (52.3 ± 3.6 vs. 38.9 ± 3.9; F = 7.27, p = .008) and greater craving reduction (58.2 ± 3.8 vs. 45.9 ± 4.2; F = 5.33,

Table 1. Study 1: Subscales of the Modified Cigarette Evaluation Questionnaire (mCEQ) by Nicotine Level

Measurement[a] (0–100 Scale)	Usual Brand Mean (*SE*)	Lower LS Means (SE)[b]	Intermediate LS Mean (*SE*)[b]	Higher LS Mean (*SE*)[b]	Lower vs. Intermediate	Lower vs. Higher	Intermediate vs. Higher
Satisfaction	83.6 (1.4)	36.8 (4.1)	54.1 (4.1)	61.4 (4.1)	$\|t\| = 3.26$*	$\|t\| = 4.65$***	$\|t\| = 1.38$
Psychological Reward	53.7 (2.2)	33.5 (3.1)	40.9 (3.1)	43.6 (3.1)	$\|t\| = 1.85$	$|t| = 2.53$$^{+}$	$\|t\| = 0.68$
Aversion	13.0 (1.7)	16.7 (2.8)	14.1 (2.8)	18.2 (2.8)	$\|t\| = 0.73$	$\|t\| = 0.40$	$\|t\| = 1.14$
Enjoyment of Sensation	73.1 (2.4)	32.7 (4.2)	49.3 (4.2)	54.8 (4.2)	$\|t\| = 2.70$*	$\|t\| = 4.09$**	$\|t\| = 1.03$
Craving Reduction	62.8 (3.3)	42.8 (4.5)	54.9 (4.5)	58.6 (4.5)	$\|t\| = 2.07$	$|t| = 2.71$$^{+}$	$\|t\| = 0.64$

Notes. LS = least square; SE = standard error.
[a]Subscale scores were averaged across the items.
[b]Adjusted for baseline response (usual brand), gender, menthol use, study site, cigarette order, interactions between nicotine level and menthol and gender, and repeated measures across subjects.
$^{+}p \le .05$. *$p \le .01$. **$p \le .001$. ***$p \le .0001$.

$p = .023$), but no significant interaction effect was observed between nicotine content and menthol status (data not shown). Women reported greater craving reduction than men (58.4±4.6 vs. 45.9±3.8; $F = 5.91$, $p = .016$). No significant gender or nicotine content by gender interactions were observed across the subscales other than craving reduction. For the Aversion subscale, participants did not score the experimental cigarettes differently and no menthol status or nicotine content by menthol status interaction effect was observed.

Other Subjective Responses to Spectrum Cigarettes

Significant differences were observed between LN and HN cigarettes for items measuring cigarette strength (30.1±4.9 vs. 46.0±4.8; $|t| = 2.54$, $p = .037$), flavorfulness (32.5±4.3 vs. 52.5±4.3; $|t| = 3.61$, $p = .001$), estimate of amount of nicotine in cigarettes (2.7±0.16 vs. 3.4±0.16; $|t| = 3.68$, $p = .001$), and liking (35.0±4.4 vs. 58.1±4.3; $|t| = 4.13$, $p = .0002$) and disliking (57.6±4.9 vs. 31.2±4.8; $|t| = 4.22$, $p = .0001$) of the experimental cigarette, with higher values assigned to the HN cigarettes except for disliking. Similar significant differences were observed between LN and IN cigarettes for liking (35.0±4.4 vs. 54.2±4.3; $|t| = 3.42$, $p = .003$) and disliking (57.6±4.9 vs. 39.5±4.8; $|t| = 2.89$, $p = .014$). Nonmenthol smokers compared with menthol smokers reported their cigarettes to be significantly more flavorful (52.4±3.7 vs. 33.5±4.0; $F = 13.64$, $p = .0003$), liked their cigarettes more (56.1±3.7 vs. 42.1±4.0; $F = 7.44$, $p = .007$), and disliked their cigarette less (33.8±4.1 vs. 51.6±4.5; $F = 9.63$, $p = .002$). No significant differences were observed for harshness of cigarette and on any of the measures between the IN and HN cigarettes. No differences between men and women were observed.

Both HN and IN cigarettes were associated with higher monetary value than LN cigarettes when subjects were asked the price at which they would switch to money over a pack of cigarettes ($4.88±0.40, $4.90±0.40, $3.44±0.41; $|t| = 2.75$, $p = .020$ for HN vs. LN and $|t| = 2.78$, $p = .019$ for IN vs. LN) and similarly for nonmenthol compared with menthol smokers ($5.15±0.34 vs. $3.66±0.38; $F = 9.58$, $p = .002$). The nicotine content by gender interaction was significant ($F = 3.147$, $p = .047$). Stratification by gender indicated that the significant differences in price by nicotine content shown above were evident among women ($5.01±0.66, $5.68±0.66, $2.88±0.66; $|t| = 2.38$, $p = .065$ for HN vs. LN and $|t| = 3.13$, $p = .009$ for IN vs. LN) but not men ($4.88±0.46, $4.15±0.47, $4.00±0.47; $|t| = 1.42$, $p = .484$ for HN vs. LN and $|t| = 0.24$, $p = 1.00$ for IN vs. LN).

When ranking the cigarettes, 95.8% and 92.3% of the menthol and nonmenthol smokers, respectively, chose their usual brand as their first choice. IN and HN cigarettes were significantly more likely to be ranked higher than LN cigarettes (Odds Ratios, *OR* = 2.5 (95% *CI*: 1.3–4.6); $|t| = 2.87$, $p = .005$ and 4.0 (95% *CI*: 2.1–7.5); $|t| = 4.37$, $p < .0001$, respectively). No differences between men and women were observed.

Perceived Health Risk

Participants scored the HN cigarettes as having a significantly higher risk of addiction than the LN and IN cigarettes (Table 2). Smokers rated the HN cigarettes as having significantly greater PHRs on all other measures relative to LN cigarettes. No significant differences in perception of health risks were observed for IN versus HN cigarettes, with the exception of risk for addiction. Significant differences were observed between LN versus IN cigarettes for lung cancer and approached significance for risk of emphysema ($p = .066$). No differences between men and women or by menthol status were observed.

Heart Rate and Blood Pressure

Systolic and diastolic blood pressure and heart rate were significantly higher for the HN compared to the LN cigarettes (120.2±1.6 vs. 117.1±1.6; $|t| = 2.45$, $p = .049$; 78.0±1.2 vs. 74.6±1.2, $|t| = 3.49$, $p = .002$; and 73.9±4.5 vs. 71.3±1.5, $|t| = 3.20$, $p = .006$). IN cigarettes revealed significantly higher diastolic blood pressure (77.7±1.2 vs. 74.6±1.2; $|t| = 3.24$, $p = .005$) and higher heart rate (73.4±1.5 vs. 71.3±1.5; $|t| = 2.51$, $p = .042$) than LN cigarettes. No significant differences were observed for other comparisons. No menthol status, gender, or menthol status or gender by nicotine content interaction effects were observed for blood pressure. The heart rates of nonmenthol smokers were borderline significantly higher than the heart rates of menthol smokers (75.3±1.8 vs. 70.4±2.0; $F = 3.90$, $p = .055$).

STUDY 2

Methods

Subjects and Cigarettes

Subject recruitment methods and cigarettes were identical to Study 1 with the following exceptions: subjects were only recruited at the University of Minnesota and smoked 10–20

Table 2. Study 1: Perceived Health Risk (PHR) by Nicotine Level

Measurement (0–100 Scale)	Usual Brand Mean (*SE*)	Lower LS Mean (*SE*)[a]	Intermediate LS Mean (*SE*)[a]	Higher LS Mean (*SE*)[a]	Lower vs. Intermediate	Lower vs. Higher	Intermediate vs. Higher
Lung cancer	79.8 (2.6)	56.6 (4.6)	66.7 (4.6)	68.3 (4.6)	$\|t\| = 2.92^{+}$	$\|t\| = 3.41$*	$\|t\| = 0.48$
Emphysema	77.6 (2.7)	60.6 (4.2)	67.9 (4.2)	70.8 (4.2)	$\|t\| = 2.33$	$\|t\| = 3.26$*	$\|t\| = 0.93$
Bronchitis	76.2 (3.2)	59.4 (4.2)	65.7 (4.2)	68.4 (4.1)	$\|t\| = 1.93$	$\|t\| = 2.76^{+}$	$\|t\| = 0.82$
Other cancers	74.5 (3.0)	57.2 (3.9)	64.0 (3.9)	68.8 (3.9)	$\|t\| = 2.02$	$\|t\| = 3.43$*	$\|t\| = 1.40$
Heart disease	77.1 (3.0)	62.6 (3.9)	67.6 (3.9)	72.3 (3.9)	$\|t\| = 1.65$	$\|t\| = 3.23$*	$\|t\| = 1.57$
Risk of addiction	85.9 (2.3)	51.0 (4.8)	58.1 (4.7)	69.9 (4.7)	$\|t\| = 1.82$	$\|t\| = 4.82$***	$\|t\| = 3.00$*
Stroke	70.9 (3.3)	58.1 (3.7)	62.8 (3.6)	68.3 (3.6)	$\|t\| = 1.63$	$\|t\| = 3.56$*	$\|t\| = 1.92$

Notes. LS = least square; SE = standard error.
[a]Adjusted for baseline response (usual brand), gender, menthol use, study site, cigarette order, interactions between nicotine level and menthol and gender, and repeated measures across subjects.
$^{+}p \le .05$. *$p \le .01$. **$p \le .001$. ***$p \le .0001$.

cigarettes/day (to reduce the number of cartons of cigarettes that would be needed).

Study Design

Subjects attended three clinic visits. For the first clinic visit, subjects were instructed to continue smoking their usual cigarette brand for 1 week and provided a daily diary to record the number of cigarettes smoked per day, a container to collect cigarette butts, and a urine cup to collect a first morning void. Cigarette butts were collected by the subject on the day before the next clinic visit 1 week later (to be analyzed later) and the urine on the day of the clinic visit.

On the second clinic visit, subjects returned their daily diary, the filled container of usual-brand cigarette butts, and the first-morning-void urine sample. Heart rate, blood pressure, and CO were obtained and subjective forms similar to those in Study 1 were completed. Subjects were randomly assigned experimental cigarettes in a double-blind manner and instructed to smoke the experimental cigarette exclusively for 1 week. Subjects were given a daily diary to record the number of study and/or usual-brand cigarettes smoked each day. Subjects collected cigarette butts on the last day of study. Subjects were asked to collect a first-morning-void urine sample on the day of the third clinic visit, which involved the same procedures as the second clinic visit.

Urine samples were analyzed for total cotinine (Murphy et al., 2004) and total nicotine equivalents (TNE), which is the sum of nicotine, cotinine, trans 3′-hydroxycotinine, and their respective glucuronide conjugates (Scherer et al., 2007).

Statistical Analysis

Demographic and smoking history data were summarized. Cigarettes smoked (both usual brand and experimental) in a given week were summed over the first 7 reported days. If the number of cigarettes smoked was missing for 1 day, the average of the other days in that week was used in its place. Outcome variables similar to Study 1 were analyzed in Study 2, except biomarker levels were also assessed (CO, total cotinine, and TNE). All outcomes were analyzed using linear regression models adjusting for baseline response (relating to their usual brand), experimental cigarette nicotine content (LN, IN, HN), gender, and nicotine content and gender interaction. Change in number of cigarettes smoked, CO, and other biomarker values from usual brand (baseline) were also assessed by cigarette type. We were not able to adjust for menthol status in this study due to small numbers in the menthol group. LS means ± *SE* were reported for each nicotine level unless otherwise noted, and *p* values were adjusted for multiple comparisons using a Bonferroni correction. All significance levels were set at .05.

Results

Thirty-six subjects were randomized to LN (n = 13), IN (n = 11), and HN (n = 12) cigarettes. One subject from the LN group was excluded because he did not smoke any experimental cigarettes. Table 3 shows the demographic and smoking history information; no significant differences were observed across cigarette types, although a trend was observed for age. Because of the small sample size, only a few key significant results will be discussed and most of the results are descriptive.

Compliance With Product

The footnote for Table 4 shows the number of usual-brand cigarettes smoked by each smoker who reported using them. Among those assigned to the LN cigarettes, five subjects (including the subject who never smoked the experimental cigarette) smoked their usual-brand cigarette during the treatment period. Among those assigned to the IN and HN cigarettes, 4 and 2 subjects, respectively, smoked their usual-brand cigarettes during the treatment week.

Cigarette and Nicotine Exposure

Subjects who were assigned to the LN cigarettes smoked significantly fewer experimental cigarettes over the course of the treatment week than those assigned to the HN cigarettes (92.1 ± 15.9 vs. 157.6 ± 15.2; $|t| = 2.97$, $p = .018$; Figure 1). Additionally, those who were assigned to the HN cigarettes smoked significantly more experimental cigarettes during the treatment week than usual-brand cigarettes during the pretreatment week (change: 33.0 ± 13.4; $|t| = 2.46$, $p = .020$). Those assigned to the LN cigarettes smoked fewer compared with their usual-brand cigarettes, although this was not statistically significant (change: −10.6 ± 14.0 cigarettes/week, $|t| = -0.76$, $p = .454$). There were no differences by gender.

Table 4 shows the biomarker values for each subject, and Table 5 shows the mean values by cigarette type assignment. Comparisons by randomization group (LN, IN, HN) found no significant differences in baseline levels of CO (20.1 ± 3.0,

Table 3. Study 2: Demographics and Smoking History of All Subjects and by Nicotine Level

	Total	Lower Nicotine	Intermediate Nicotine	Higher Nicotine	
Variables		*N*(%)			*p* Value
Total	35	12 (34.3)	11 (31.4)	12 (34.3)	
Gender					
Male	15 (42.9)	4 (33.3)	4 (36.4)	7 (58.3)	.477
Female	20 (57.1)	8 (66.7)	7 (63.6)	5 (41.7)	
Ethnicity					
Hispanic or Latino	2 (5.7)	1 (8.3)	0 (0.0)	1 (8.3)	1.00
Not Hispanic/Latino	33 (94.9)	11 (91.7)	11 (100.0)	11 (91.7)	
Race					
American Indian/Alaskan native	1 (2.9)	1 (8.3)	0 (0.0)	0 (0.0)	.585
Asian	1 (2.9)	0 (0.0)	1 (9.1)	0 (0.0)	
Black or African American	7 (20.0)	3 (25.0)	1 (9.1)	3 (25.0)	
White	22 (62.9)	8 (66.7)	7 (63.6)	7 (58.3)	
More than one race	4 (11.4)	0 (0.0)	2 (18.2)	2 (16.7)	
Smoke menthols					
No	24 (68.6)	8 (66.7)	8 (72.7)	8 (66.7)	1.00
Yes	11 (31.4)	4 (33.3)	3 (27.3)	4 (33.3)	

	N	Mean (*SD*)	*N*	Mean (*SD*)	*N*	Mean (*SD*)	*N*	Mean (*SD*)	*p* Value
Age (y)	35	37.9 (11.6)	12	37.0 (12.4)	11	32.7 (10.8)	12	43.6 (9.6)	.072
Highest grade completed	35	13.4 (2.4)	12	13.8 (1.1)	11	13.9(1.9)	12	12.6 (3.5)	.370
Cigarettes/day	35	17.2 (4.4)	12	16.8 (5.1)	11	16.1 (3.5)	12	18.7 (4.3)	.351
Years daily smoking	35	17.9 (11.0)	12	14.8 (10.9)	11	15.4 (11.7)	12	23.3 (9.0)	.104

16.4±2.8, 17.0±2.5; $F = 0.47$, $p = .629$), total cotinine (22.2±4.7, 21.0±4.5, 19.2±4.0; $F = 0.13$, $p = .881$), or TNE (90.3±15.6, 71.4±14.8, 74.4±13.5; $F = 0.45$, $p = .644$).

Among subjects who smoked three or fewer of their usual-brand cigarettes during the treatment week prior to urine collection ($n = 32$), subjects smoking the HN and IN cigarettes had higher CO levels than those smoking LN cigarettes, although this difference was only borderline significant for the IN cigarettes ($p = .068$). There were no significant differences in CO levels between the IN and HN cigarettes. In comparison with their baseline (usual brand) CO levels, those smoking the LN cigarettes had a significant decrease in CO levels (change: 7.8±2.8; $|t| = 2.77$, $p = .010$), whereas CO did not change significantly in the IN and HN conditions compared with baseline (change: −2.0±2.6; $|t| = 0.74$, $p = .465$ and −2.7±2.4; $|t| = 1.12$, $p = .273$, respectively).

Among those with available biomarker data and who smoked three or fewer usual-brand cigarettes during the treatment week ($n = 31$), total cotinine and TNE were significantly lower in subjects using the LN cigarettes than those using the HN cigarettes (Table 5). In comparison to baseline, those smoking the LN cigarettes had significantly lower total cotinine and TNE levels at the end of treatment (change: −16.2±5.0; $|t| = 3.24$, $p = .003$ and −64.5±14.5; $|t| = 4.46$, $p = .0002$, respectively), and those smoking the IN cigarettes had borderline significantly lower cotinine levels (change: −7.6±4.7; $|t| = 1.62$, $p = .117$) and significantly lower TNE levels (change: −33.0±13.5; $|t| = 2.44$, $p = .022$). Total cotinine and TNE levels among those smoking HN cigarettes were not significantly different from baseline (change: 1.3±4.4; $|t| = 0.31$, $p = .761$ and −14.7±12.7; $|t| = 1.16$, $p = .258$, respectively). There were no differences by gender.

Modified Cigarette Evaluation Questionnaire

Significant differences were observed between the LN and HN cigarette conditions for satisfaction (8.3±7.5 vs. 47.8±7.2; $|t| = 3.71$, $p = .003$) and Enjoyment of Sensation (13.8±7.5 vs. 41.3±7.0; $|t| = 2.63$, $p = .041$). A trend was observed for Psychological Reward (28.5±6.2 vs. 47.8±6.1; $|t| = 2.22$, $p = .105$). All other comparisons between nicotine content cigarette conditions and mCEQ outcomes were not statistically significant. There were also no differences by gender.

Other Subjective Responses to Spectrum Cigarettes

Smokers assigned the HN cigarettes compared with LN and IN conditions reported greater liking (45.2±7.6 vs. 7.1±8.0 and 16.7±8.1; $|t| = 3.44$, $p = .006$ and $|t| = 2.58$, $p = .046$, respectively) and significantly or nearly significantly lower disliking of the cigarettes (43.5±7.9 vs. 91.5±8.4 and 72.6±8.7; $|t| = 4.15$, $p = .001$ and $|t| = 2.49$, $p = .058$, respectively). Consistent with the subjective ratings, participants in the HN condition indicated that they would switch to money over cigarettes at a higher monetary value than subjects in the LN condition ($5.44±0.69 vs. $1.68±0.72, $|t| = -3.79$, $p = .002$). There were no differences in these outcomes by gender.

DISCUSSION

The results from these studies indicated that these research cigarettes were generally distinguishable and produced a dose–response effect. Smokers, blind to cigarette type, were able to discriminate cigarettes varying in nicotine content, particularly between the LN and HN and LN and IN doses, with the LN

Table 4. Study 2: Levels of Urinary Cotinine and Total Nicotine Equivalents for Each Subject by Cigarette Type

	Cotinine (nmol/ml)		Total nicotine equivalent (nmol/ml)	
Cigarette type	Usual cigarettes	Experimental cigarettes	Usual cigarettes	Experimental cigarettes
Subject Number				
Lower nicotine				
202	14.0	4.7	72	17
204	6.7	0.1	58	1
214*	8.8	1.7	25	5
221*	22.6	5.0	67	16
231	41.3	0.5	123	2
232	36.3	17.0	170	69
234	16.3	0.1	62	LOD
235	17.7	8.8	115	56
238	59.7	13.7	195	38
240*	8.6	14.0	24	48
246	42.1	Missing	162	Missing
250*	14.0	0.8	31	2
Intermediate nicotine				
205*	25.3	2.4	78	8
210	25.0	6.6	83	19
211*	34.2	10.3	70	24
216*	1.3	1.9	6	12
225*	44.7	37.3	107	93
227	14.7	14.6	91	66
230	3.2	2.1	18	12
236	24.5	7.0	52	18
239	17.5	2.3	51	7
244	7.6	36.6	82	88
248	14.5	7.9	63	29
Higher nicotine				
206	3.4	4.0	41	41
209	39.8	14.1	193	45
213	20.6	24.3	65	61
215*	28.7	38.8	91	85
222	7.5	16.3	26	68
226*	10.0	23.0	94	72
233	8.1	24.1	60	58
237	29.4	11.2	104	56
242	29.5	Missing	62	Missing
245	18.3	26.6	44	69
252	21.4	14.0	51	41
254	14.5	20.3	42	62

Note. *Self-reported usual-brand cigarettes smoked during treatment period: 214 and 221 smoked usual-brand cigarettes after urine collection, 240 = 13 cigarettes, 250 = 1 cigarette, 205 = 3 cigarettes, 211 = 2 cigarettes, 216 = 7 cigarettes, 225 = 1 cigarette, 215 smoked after urine collection, 226 = 1 cigarette; LOD = limit of detection.

cigarettes producing a less favorable subjective response. Only two variables led to a distinction between the IN and HN cigarettes in the expected direction, "risk for addiction" in Study 1 and "liking" in Study 2. Cotinine, TNE, and CO levels significantly decreased during Study 2 following the LN cigarettes (compared with baseline) and TNE decreased following the IN cigarettes. No significant changes on any of these exposure measures were observed for the HN dose compared with usual brand, although these subjects smoked significantly higher number of cigarettes. Significant differences between the HN and LN conditions were observed for CO, total cotinine, and TNE.

Prior studies also observed that smokers can discriminate subjectively across differing nicotine content cigarettes (Benowitz et al., 2007, 2012; Benowitz, Jacob, & Herrera, 2006; Hatsukami, Kotlyar et al., 2010), particularly between higher versus lower nicotine content cigarettes. In an acute dosing study, subjects were asked to smoke one of their usual-brand cigarette and then on five separate occasions to smoke a research cigarette that varied in nicotine content (from 0.6 to 10.1 mg nicotine content per cigarette or 0.13 to 0.89 mg Federal Trade Commission (FTC) determined nicotine yield; Benowitz, Jacob, & Herrera 2006). Lower nicotine content cigarettes were rated as less strong, much too smooth, poorer

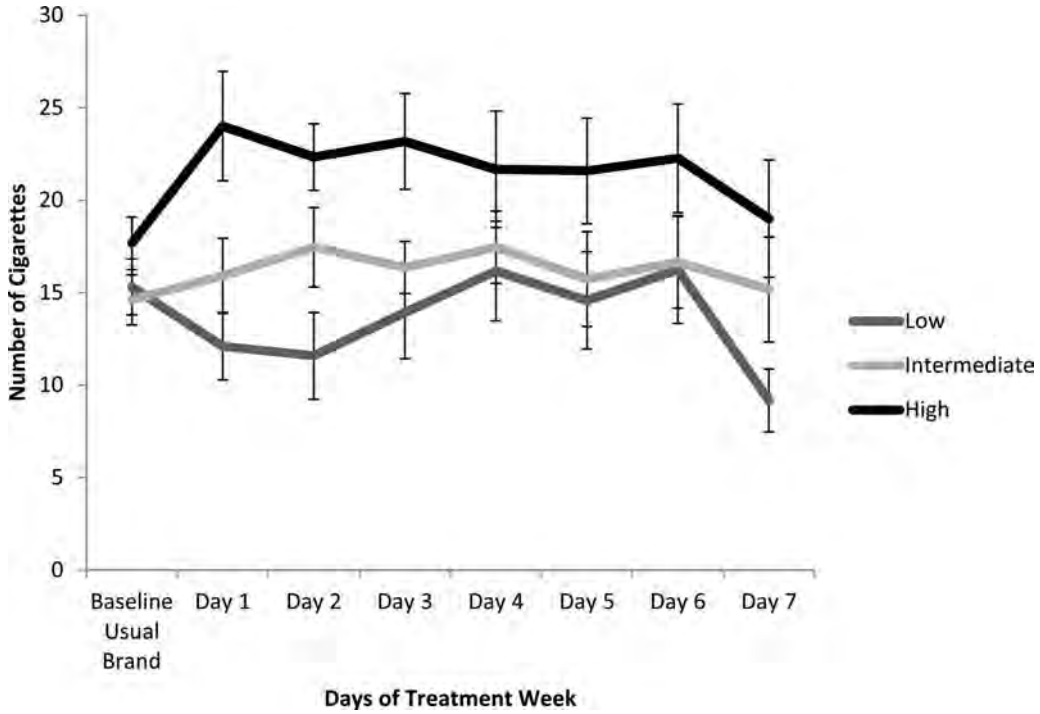

Figure 1. Means and standard errors (*SE*) of number of usual and experimental cigarettes smoked by nicotine level.

Table 5. Study 2: Biomarker Levels After 1 Week of Product Use by Nicotine Level, Adjusted for Baseline Levels and Gender

Measurement	Usual Brand Mean (*SE*)	Lower LS Mean (*SE*)	Intermediate LS Mean (*SE*)	Higher LS Mean (*SE*)	Lower vs. Intermediate	Lower vs. Higher	Intermediate vs. Higher
Cotinine (nmol/ml)	21.8 (2.3)	5.0 (3.5)	13.4 (3.2)	20.3 (3.1)	$\|t\| = 1.77$	$\|t\| = 3.31$*	$\|t\| = 1.55$
Nicotine equivalents (nmol/ml)	81.6 (7.8)	19.9 (8.1)	40.1 (7.6)	60.8 (7.1)	$\|t\| = 1.81$	$\|t\| = 3.79$*	$\|t\| = 2.01$
Carbon monoxide	17.1 (1.4)	11.3 (2.3)	18.9 (2.1)	19.9 (1.9)	$\|t\| = 2.43$	$\|t\| = 2.91$**	$\|t\| = 0.38$

Notes. LS = least square; *SE* = standard error.
Excludes three subjects who smoked more than three usual-brand cigarettes during the study period.
*$p \le .01$. **$p \le .05$.

quality, less satisfying, and having less nicotine. The greatest differences were observed with the 1 mg (0.13 mg nicotine yield) compared with 8 mg (0.63 mg nicotine yield) and 12 mg (0.89 mg nicotine yield) nicotine content cigarettes. The 2 mg (0.18 mg nicotine yield) and 4 mg (0.33 nicotine yield) nicotine content cigarettes occasionally showed differences compared with the higher nicotine content cigarettes. No differences were observed among 1, 2, and 4 mg nicotine content cigarettes (<0.33 nicotine yield). In another study, Benowitz and colleagues (2007) examined the effects of a progressive weekly reduction in nicotine content in cigarettes (12, 8, 4, 2, and 1 mg nominal nicotine content or 0.8, 0.6, 0.3, 0.2, and 0.1 mg FTC determined nicotine yield). With regards to subjective measures, no change was observed in the Profile of Mood Scale score or Center for Epidemiologic Studies Depression Scale depression rating. However, withdrawal scores for irritability and increased eating were significantly higher at week 6 while smoking 1 mg nicotine content cigarette (0.1 mg nicotine yield) compared with baseline while smoking usual brand. No direct comparisons were described between the different doses of cigarettes for these measures and measures of cigarette acceptance, although subjects reported that the reduced-nicotine cigarettes were less strong, less flavorful, of generally lower quality and less satisfying compared with their usual-brand cigarettes. The third study involved a progressive reduction in nicotine content in cigarettes at monthly intervals using similar nicotine content cigarettes as the prior weekly reduction study (0.9, 0.6, 0.4, 0.2, and 0.1 mg nicotine yield). Increased confusion and decreased vigor were observed when smokers switched from their usual-brand cigarettes to cigarettes that were equal to or less than 4 mg nicotine content (or 0.4 mg nicotine yield). Similar to the prior study, the reduced-nicotine content cigarettes were rated as milder, less satisfying, having lower nicotine effect, and of lesser quality than usual-brand cigarettes. No other details comparing the varying nicotine content cigarettes were provided. In a study conducted by Hatsukami, Kotylar, and colleagues (2010), rather than a progressive reduction in nicotine content, subjects were asked to switch completely to

0.05 or 0.3 mg nicotine yield cigarettes or to nicotine lozenge for 6 weeks. Risk for addiction was perceived to be significantly lower for the 0.05 mg compared with the 0.3 mg nicotine cigarette. The results from these previous studies are similar to our finding that the greatest differences in subjective responses occur between a very LN content cigarette (most likely <0.1 mg nicotine yield) compared with a substantially higher nicotine content cigarette (>0.3 or 0.4 mg nicotine yield).

Reductions in smoking behavior and exposure to cotinine, nicotine, and CO is typically observed with the lower nicotine content cigarettes. In the acute cigarette dosing study conducted by Benowitz and colleagues (2006), a significant dose–response relationship was observed between intake of nicotine and machine determined nicotine yield (and nicotine content), although actual exposure was generally greater than predicted from machine determined nicotine yield of the cigarettes. Compensation was lower in the 1 mg cigarette (0.13 mg nicotine yield) compared with the 4 mg cigarette (0.33 nicotine yield; 19% vs. 38%, respectively) and to the 8 mg (0.63 mg nicotine yield) nicotine cigarette (64%); although these differences were not significant, they suggest that compensation may be observed less at very low yields. In the Benowitz and colleagues (2007) study where subjects underwent a weekly progressive decrease in nicotine content, no significant change was observed for smoking behavior or CO during nicotine reduction, but a progressive decrease occurred for cotinine levels. In the Benowitz and colleagues (2012) study where subjects underwent a monthly decrease in nicotine content, no significant change in smoking behavior was observed until smokers were switched to 1 mg nicotine content cigarette (or 0.1 mg nicotine yield) at which point smoking rate declined. A significant decrease in cotinine was observed after switching to 4 mg (or 0.4 mg nicotine yield) nicotine cigarette. By the end of the study at week 26 (1 mg nicotine content), cotinine levels were 30% of the baseline value among those who complied with use of the cigarettes. With regard to the Hatsukami, Kotlyar, and colleagues (2010) study, where subjects reduced to lower nicotine content cigarettes immediately, an increased number of cigarettes and CO were observed for the 0.3 mg nicotine yield cigarette relative to baseline, but these measures decreased for the 0.05 mg cigarette, resulting in significant or near significant differences between the two cigarette yields. Cotinine was significantly reduced in both cigarette conditions with greatest reductions in those assigned to the 0.05 mg nicotine cigarette. The results from these studies are concordant with our findings that smokers are sensitive to nicotine contents of the cigarettes and tend to show decreases in smoking behavior and exposure at the lowest nicotine content. Furthermore, significant changes in subjective responses are also likely to occur at nicotine yields <0.1 mg. Increased smoking with or without increases in exposure (e.g., CO) may occur at more intermediate doses of nicotine, but whether this increase has a significant impact on health is unknown and will require more investigation.

Three additional results are of interest. Less compliance was observed with the LN and IN than HN cigarettes. These results are similar to those observed by Hatsukami, Kotylar, and colleagues (2010), in which the lower dose was modestly associated with more subjects smoking usual-brand cigarettes. With regards to differences in menthol versus nonmenthol cigarettes, smokers of menthol cigarettes did not report satisfaction or liking their cigarettes as much as the nonmenthol smokers. It is possible that switching to cigarettes that differed in both menthol and nicotine content levels compared with their usual brands led to more dissatisfaction with these cigarettes or that menthol smokers tended to smoke higher nicotine content cigarettes. Efforts to manufacture menthol cigarettes that are equally palatable as nonmenthol cigarettes may be important. Finally, gender differences were only observed in Study 1 and for craving reduction and monetary value of cigarettes. Although these results are suggestive, due to the small sample size, further research is required before any conclusions can be made.

In summary, this study showed that the dose–response results with the Spectrum research cigarettes are similar to those observed in prior studies that compared cigarettes varying in nicotine content. In general, very LN content cigarettes (especially <0.1 mg nicotine yield) tend to lead to reduced smoking and significant differences in subjective responses compared with cigarettes with higher nicotine yields (>0.4 or 0.3 mg nicotine yield cigarettes).

FUNDING

This work was supported by the National Institute on Drug Abuse at the National Institutes of Health Intramural Research Program and under contract no. HHSN271201000003C, U54DA031659, and the University of Minnesota, Forster Family Professor in Cancer Prevention Endowment.

DECLARATION OF INTERESTS

Dorothy Hatsukami was funded by Nabi Biopharmaceuticals and NIDA to be a site for a nicotine immunotherapy trial. There are no other declarations.

ACKNOWLEDGMENTS

We appreciate the contributions of Kathy Longley for her assistance on the manuscript. Special thanks to Drs. Kenneth Davis and Poonam Pande at RTI for their role in making these cigarettes available to researchers.

REFERENCES

Benowitz, N. L., Dains, K. M., Hall, S. M., Stewart, S., Wilson, M., Dempsey, D., & Jacob, P., 3rd. (2012). Smoking behavior and exposure to tobacco toxicants during 6 months of smoking progressively reduced nicotine content cigarettes. *Cancer Epidemiology, Biomarkers & Prevention*, *21*, 761–769. doi:10.1158/1055–9965.EPI-11–0644

Benowitz, N. L., Hall, S. M., Stewart, S., Wilson, M., Dempsey, D., & Jacob, P., 3rd. (2007). Nicotine and carcinogen exposure with smoking of progressively reduced nicotine content cigarette. *Cancer Epidemiology, Biomarkers & Prevention*, *16*, 2479–2485. doi:10.1158/1055–9965.EPI-07-0393

Benowitz, N. L., & Henningfield, J. E. (1994). Establishing a nicotine threshold for addiction. The implications for tobacco regulation. *The New England Journal of Medicine*, *331*, 123–125.

Benowitz, N. L., Jacob, P., 3rd, & Herrera, B. (2006). Nicotine intake and dose response when smoking reduced-nicotine content cigarettes. *Clinical Pharmacology and Therapeutics*, *80*, 703–714. doi:10.1016/j.clpt.2006.09.007

Cappelleri, J. C., Bushmakin, A. G., Baker, C. L., Merikle, E., Olufade, A. O., & Gilbert, D. G. (2007). Confirmatory factor analyses and reliability of the modified cigarette evaluation questionnaire. *Addictive Behavior*, *32*, 912–923. doi:10.1016/j.addbeh.2006.06.028

Doll, R., Peto, R., Boreham, J., & Sutherland, I. (2004). Mortality in relation to smoking: 50 years' observations on male British doctors. *BMJ*, *328*, 1519. doi:10.1136/bmj.38142.554479.AEbmj.38142.554479.AE [pii]

Gray, N., Henningfield, J. E., Benowitz, N. L., Connolly, G. N., Dresler, C., Fagerstrom, K., . . . Boyle, P. (2005). Toward a comprehensive long term nicotine policy. *Tobacco Control*, *14*, 161–165. doi:10.1136/tc.2004.010272

Hatsukami, D., Kotlyar, M., Hertsgaard, L. A., Zhang, Y., Carmella, S. G., Jensen, J. A., . . . Hecht, S. S. (2010). Reduced nicotine content cigarettes: effects on toxicant exposure, dependence and cessation. *Addiction*, *105*, 343–355. doi:10.1111/j.1360-0443.2009.02780.x

Hatsukami, D., Perkins, K. A., Lesage, M. G., Ashley, D. L., Henningfield, J. E., Benowitz, N. L., . . . Zeller, M. (2010). Nicotine reduction revisited: science and future directions. *Tobacco Control*, *19*, e1–e10. doi:10.1136/tc.2009.035584

Jacobs, E. A., & Bickel, W. K. (1999). Modeling drug consumption in the clinic using simulation procedures: demand for heroin and cigarettes in opioid-dependent outpatients. *Experimental and Clinical Psychopharmacology*, *7*, 412–426.

Murphy, S. E., Link, C. A., Jensen, J., Le, C., Puumala, S. S., Hecht, S. S., . . . Hatsukami, D. (2004). A comparison of urinary biomarkers of tobacco and carcinogen exposure in smokers. *Cancer Epidemiology, Biomarkers and Prevention*, *13*, 1617–1623.

Peto, R., Lopez, A. D., Boreham, J., Thun, M., & Heath, C. Jr. (1992). Mortality from tobacco in developed countries: Indirect estimation from national vital statistics. *Lancet*, *339*, 1268–1278.

Scherer, G., Engl, J., Urban, M., Gilch, G., Janket, D., & Riedel, K. (2007). Relationship between machine-derived smoke yields and biomarkers in cigarette smokers in Germany. *Regulatory Toxicology and Pharmacology*, *47*, 171–183. doi:10.1016/j.yrtph.2006.09.001

U.S. Department of Health and Human Services. (1988). *The health consequences of smoking: Nicotine and addiction. A report of the Surgeon General.* Rockville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, Center for Health Promotion and Education, Office on Smoking and Health.

U.S. Department of Health and Human Services. (2010). *How tobacco smoke causes disease: The biology and behavioral basis for smoking-attributable disease. A report of the surgeon general.* Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office of Smoking and Health.

Westman, E. C., Levin, E. D., & Rose, J. E. (1992). Smoking while wearing the nicotine patch: Is smoking satisfying or harmful? *Clinical Research*, *40*, 871A.

Zeller, M., Hatsukami, D., & Strategic Dialogue on Tobacco Harm Reduction Group. (2009). The Strategic Dialogue on Tobacco Harm Reduction: A vision and blueprint for action in the US. *Tobacco Control*, *18*, 324–332. doi:10.1136/tc.2008.027318f

Electronic Cigarette Use and Progression From Experimentation to Established Smoking

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abstract

BACKGROUND: It has been shown that never-smoking adolescents who try electronic cigarettes (e-cigarettes) are at increased risk of subsequent conventional cigarette smoking. We evaluated associations between e-cigarette use and progression to established smoking among adolescents who had already tried cigarettes.

METHODS: Among participants (age 12–17 years) in the nationally representative Population Assessment of Tobacco and Health survey who had smoked a cigarette (≥1 puff) but not yet smoked 100 cigarettes (N = 1295), we examined 3 outcomes at 1-year follow-up as a function of baseline e-cigarette use: (1) having smoked ≥100 cigarettes (established smoking), (2) smoking during the past 30 days, and (3) both having smoked ≥100 cigarettes and past 30-day smoking (current established smoking). Survey-weighted multivariable logistic regression models were fitted to obtain odds ratios (ORs) and 95% confidence intervals (CIs) adjusted for smoking risk factors.

RESULTS: Versus e-cigarette never use, having ever used e-cigarettes was positively associated with progression to established cigarette smoking (19.3% vs 9.7%), past 30-day smoking (38.8% vs 26.6%), and current established smoking (15.6% vs 7.1%). In adjusted models, e-cigarette ever use positively predicted current established smoking (OR: 1.80; 95% CI: 1.04–3.12) but did not reach statistical significance (α = .05) for established smoking (OR: 1.57; 95% CI: 0.99–2.49) and past 30-day smoking (OR: 1.32; 95% CI: 0.99–1.76).

CONCLUSIONS: Among adolescent cigarette experimenters, using e-cigarettes was positively and independently associated with progression to current established smoking, suggesting that e-cigarettes do not divert from, and may encourage, cigarette smoking in this population.

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DOI: https://doi.org/10.1542/peds.2017-3594

Accepted for publication Jan 22, 2018

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WHAT'S KNOWN ON THIS SUBJECT: In previous studies of youth who have never smoked cigarettes, those who tried electronic cigarettes (e-cigarettes) were more likely to initiate conventional cigarette smoking compared with e-cigarette never users. In cross-sectional studies, e-cigarette use is associated with established youth smoking.

WHAT THIS STUDY ADDS: Among youth who already experimented with cigarettes but were not yet established smokers, having used e-cigarettes was prospectively associated with onset of current established cigarette smoking. For these youth, e-cigarettes appear to encourage progression to established smoking.

To cite: Chaffee BW, Watkins SL, Glantz SA. Electronic Cigarette Use and Progression From Experimentation to Established Smoking. *Pediatrics.* 2018;141(4):e20173594

Electronic cigarettes (e-cigarettes) are increasingly popular among youth; from 2014 to 2016, more US middle and high school students used e-cigarettes than any other tobacco product, including conventional cigarettes.[1] All currently available longitudinal studies have revealed that among never-smoking adolescents and young adults, e-cigarette use is associated with subsequent cigarette smoking.[2–6] This association was shown in studies taking place in California,[3] Hawaii,[2] and the Mid-Atlantic region,[7] as well as in nationally representative US samples,[6,8] Canada,[4] and the United Kingdom.[5] Seven of these studies were summarized in a recent meta-analysis, revealing more than a threefold increase in the risk of cigarette smoking initiation when comparing youth e-cigarette ever users to never users.[9] Although this association between e-cigarettes and smoking initiation has been consistent across the literature and could be explained by using a proposed "catalyst" model,[10] some have argued that the relationship partly reflects a shared propensity for experimentation with different nicotine-containing products.[11]

Many individuals at low risk of smoking initiation may be included in studies of baseline cigarette never-users. In contrast, youth who have already begun cigarette experimentation represent a population at high risk of progression to greater levels of cigarette use later in adolescence and into adulthood. Although smoking even 1 cigarette is concerning, becoming an established smoker in adolescence is of substantial clinical and public health concern and is strongly associated with continuing to smoke regularly.[12] Therefore, in the present investigation we consider high-risk youth, as evident by having already tried smoking (ever smoked ≥1 puff) but not yet smoked 100 cigarettes, and evaluate whether e-cigarette use in this population predicts progression from experimentation to established cigarette smoking 1 year later.

In a previous cross-sectional analysis of the 2011 and 2012 National Youth Tobacco Surveys (NYTSs), among youth who had ever smoked a cigarette, ever use of e-cigarettes was associated with being an established smoker (lifetime smoked ≥100 cigarettes), including after adjusting for socio-demographic variables.[13] However, the cross-sectional design of that analysis precluded causal conclusions because of uncertain temporal sequencing between e-cigarette use and established smoking.

In the current study, we used the nationally representative Population Assessment of Tobacco and Health (PATH) Study[14] Waves 1 (2013–2014) and 2 (2014–2015) to examine these same relationships prospectively. We hypothesized that among PATH youth participants who had already tried cigarette smoking but not yet smoked a total of 100 cigarettes, use of e-cigarettes would be positively associated with becoming an established cigarette smoker within 1 year.

METHODS

Researchers from the PATH Study selected participants using a 4-stage stratified probability design with oversampling for tobacco users, African Americans, and young adults (ages 18–24 years). The PATH youth sample consisted of adolescents (up to 2 per household) whose parents were selected for the PATH adult sample.[14] Researchers from the PATH Youth Study enrolled 13 651 US adolescents ages 12 to 17 years at baseline (2013–2014), with 87.9% retention (unweighted) at Wave 2 (2014–2015).

In-home in-person computer-assisted interviews were conducted in administering the PATH questionnaire. In separate sections, participants were asked about their tobacco use (eg, ever use, number of lifetime uses, and number of days used in the past 30 days) for 8 types of tobacco and nicotine-containing products, including cigarettes and e-cigarettes. Tobacco use questions were repeated during the Wave 2 interview, including for individuals who reached age 18 before follow-up and were therefore administered the Wave 2 adult questionnaire.

In the present analysis, we included youth who had smoked ≥1 cigarette puff but had not yet smoked 100 cigarettes at baseline (smoking experimenters), with known smoking status at follow-up (N = 1295). We examined 3 outcomes at follow-up as a function of baseline e-cigarette use: (1) having smoked ≥100 cigarettes (established smoking), (2) smoking during the past 30 days, and (3) both having smoked ≥100 cigarettes and past 30-day smoking (current established smoking). We categorized e-cigarette use in 2 ways: (1) ever use or never use, and (2) never use, nonpast 30-day use (former use), or past 30-day use.

Logistic regression models (Stata 14; StataCorp, College Station, TX) were used to adjust for hypothesized confounding variables in 3 stages. First, 6 separate unadjusted models were fitted to cover each combination of independent variable (Wave 1 e-cigarette never or ever use and e-cigarette never or former or past 30-day use) and dependent variable (Wave 2 established smoking, current smoking, and current established smoking). In the second stage, we added sex, age (in years), and race and/or ethnicity (Hispanic and/or Latino, non-Hispanic white, non-Hispanic African American, other) as covariables in all models, matching the confounders used in a previous cross-sectional analysis of NYTS data.[13] In the third stage, parent education (≥1 parent with a

TABLE 1 Progression From Cigarette Experimentation to Established Smoking, According to Baseline E-cigarette Use

	n	Weighted % With Outcome	Unadjusted		Adjusted[a]		Adjusted[b]	
			OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Outcome: smoked 100 cigarettes								
Wave 1 predictors								
E-cigarette never	646	9.7	Reference	—	Reference	—	Reference	—
E-cigarette ever	582	19.3	2.23 (1.55–3.21)	<.001	2.07 (1.41–3.04)	<.001	1.57 (0.99–2.49)	.05
E-cigarette never	646	9.7	Reference	—	Reference	—	Reference	—
E-cigarette former	406	18.6	2.13 (1.43–3.18)	<.001	2.04 (1.33–3.12)	.001	1.55 (0.94–2.56)	.09
E-cigarette in the past 30 d	171	21.5	2.56 (1.58–4.14)	<.001	2.22 (1.31–3.74)	.003	1.69 (0.93–3.05)	.08
Outcome: smoked during the past 30 d								
Wave 1 predictors								
E-cigarette never	699	26.6	Reference	—	Reference	—	Reference	—
E-cigarette ever	596	38.8	1.75 (1.35–2.27)	<.001	1.65 (1.26–2.15)	<.001	1.32 (0.99–1.76)	.06
E-cigarette never	699	26.6	Reference	—	Reference	—	Reference	—
E-cigarette former	415	36.1	1.56 (1.15–2.12)	.004	1.48 (1.09–2.02)	.01	1.20 (0.86–1.68)	.29
E-cigarette in the past 30 d	176	45.3	2.29 (1.64–3.19)	<.001	2.10 (1.47–2.99)	<.001	1.64 (1.12–2.41)	.01
Outcome: smoked 100 cigarettes and smoked during the past 30 d								
Wave 1 predictors								
E-cigarette never	644	7.1	Reference	—	Reference	—	Reference	—
E-cigarette ever	580	15.6	2.43 (1.55–3.80)	<.001	2.23 (1.39–3.59)	<.001	1.80 (1.04–3.12)	.03
E-cigarette never	644	7.1	Reference	—	Reference	—	Reference	—
E-cigarette former	406	15.5	2.41 (1.46–3.97)	<.001	2.29 (1.35–3.89)	.002	1.85 (1.02–3.36)	.04
E-cigarette in the past 30 d	171	16.3	2.56 (1.52–4.32)	<.001	2.19 (1.24–3.88)	.007	1.76 (0.92–3.37)	.09

ORs and CIs corresponding to model covariates are shown in Supplemental Table 2. —, not applicable.

[a] Model covariates include the following: sex, age, and race and/or ethnicity.

[b] Model covariates additionally include the following: parent education, urban residence, household tobacco use, alcohol ever use, tobacco advertisement receptivity, sensation-seeking score, cigarette warning label exposure, interview time of year, and ever use of any other tobacco product.

bachelor degree or greater), urban residence (based on sampling units), household tobacco use (lives with ≥1 tobacco user), alcohol ever use, tobacco advertisement receptivity[15] (can recall brand of favorite advertisement), sensation-seeking score (scale from 3 to 15), cigarette warning label exposure (Likert-type scale), interview time of year (summer versus all other months), and ever use of any other tobacco product (ie, cigars, pipes, hookah, bidis, kreteks, snus, dissolvable tobacco, and conventional moist snuff or chewing smokeless tobacco) were also included in additional adjusted models. A sensation-seeking score was a composite of 3 Likert-type items (liking frightening things, willingness to break rules, and preference for exciting and unpredictable friends) and has been shown to correlate with youth tobacco use.[16] Interview time of year was included because, for youth, the scholastic calendar may play a role both in opportunity and social pressure to experiment with tobacco products.

All models were weighted for sampling design and nonresponse by using balanced repeated replication to be representative of the Wave 1 target population.[17] Multiple imputation was performed for missing observations (0.7% of data), with variance estimates adjusted accordingly.

An institutional review board at the University of California, San Francisco reviewed and designated the study protocol exempt for this analysis of deidentified survey data. The PATH Study protocol received a National Institutes of Health Certificate of Confidentiality and approval from the Westat Institutional Review Board. Parental consent was requested on behalf of participating youth. Youth who completed the questionnaire were given $25.

RESULTS

Among baseline cigarette experimenters (mean age: 15.5 years; 48.3% girls), having ever used e-cigarettes was positively associated with progression to established cigarette smoking in Wave 2 (Table 1). Compared with e-cigarette never users, e-cigarette ever users were twice as likely to report Wave 2 established smoking (19.3% vs 9.7%; $P < .001$) and current established smoking (15.6% vs 7.1%; $P < .001$) and were more likely to report past 30-day smoking (38.8% vs 26.6%; $P < .001$).

In models adjusted for sex, age, and race and/or ethnicity (Table 1), Wave 1 e-cigarette ever use (versus never use) was associated with approximately twice the odds of progression to Wave 2 established cigarette smoking (odds ratio [OR]: 2.23; 95% confidence interval [CI]: 1.55–3.21; $P < .001$), past 30-day smoking (OR: 1.75; 95% CI: 1.35–2.27; $P < .001$), and current

established smoking (OR: 2.43; 95% CI: 1.55–3.80; $P < .001$). Associations were attenuated in fully adjusted models (Table 1), but e-cigarette ever use remained a positive and statistically significant predictor of current established smoking (OR: 1.80; 95% CI: 1.04–3.12; $P = .035$). Associations did not reach the threshold for statistical significance for established smoking (OR: 1.57; 95% CI: 0.99–2.49; $P = .055$) and past 30-day smoking (OR: 1.32; 95% CI: 0.99–1.76; $P = .059$).

When baseline e-cigarette former use (tried but not used in past 30 days) and past 30-day use were considered separately, there was a stepwise increase in the probability of progression to future established smoking from never to former to past 30-day e-cigarette use (Table 1). For example, the probability of Wave 2 past 30-day cigarette smoking rose from baseline e-cigarette never use (26.6%) to former use (36.1%) to past 30-day use (45.3%). Both e-cigarette former use and past 30-day use remained statistically significantly associated with all 3 Wave 2 cigarette outcomes in models adjusted for sex, age, and race and/or ethnicity (Table 1). In fully adjusted models, baseline e-cigarette former use remained a statistically significant predictor of progression to current established smoking (OR: 1.85; 95% CI: 1.02–3.36; $P = .042$), and baseline e-cigarette past 30-day use statistically significantly predicted progression to past 30-day smoking (OR: 1.64; 95% CI: 1.12–2.41; $P = .010$). Adjustment variables that were consistently associated with greater progression to established smoking included household tobacco use and tobacco advertisement receptivity (Supplemental Table 2).

DISCUSSION

In this study, among youth who had experimented with cigarettes but had not progressed to established smoking, additional use of e-cigarettes was positively associated with future onset of current established smoking. Across 3 different definitions of established smoking and 2 different specifications of e-cigarette use, baseline e-cigarette users were at 1.5 to 2 times greater odds of progression to established smoking than e-cigarette never users, after adjustment for confounding variables. Fully adjusted associations with e-cigarette ever use were statistically significant for 1 definition of established smoking (current established smoking; $P = .035$) but fell just short of the a priori threshold for statistical significance for established smoking ($P = .055$) and past 30-day smoking ($P = .059$). The ORs in the present longitudinal analysis were in the same direction but smaller in magnitude than in the previous cross-sectional analysis of NYTS data[13] in which new trials of e-cigarettes among previously established smokers could have been captured.

Regardless of how Wave 1 e-cigarette and Wave 2 smoking variables were specified, positive associations persisted after statistical adjustment for sex, age, and race and/or ethnicity. Adding the full set of confounding variables to models, such as household tobacco use, warning label exposure, and baseline use of other tobacco products, reduced the strength of some of the observed associations to below the threshold for statistical significance. However, all associations remained positive in direction and similar in magnitude across different definitions of e-cigarette exposure and the smoking outcome.

Suggested in these results is that e-cigarette use is more likely to encourage youth smoking than to divert youth from smoking when considering individuals who have already experimented with cigarette use. Unlike adults, particularly cigarette smokers, who commonly report a desire to quit smoking as a main motivator for e-cigarette use,[18] youth are more likely to cite curiosity as a reason to try e-cigarettes.[19] E-cigarette use was not associated with cigarette quit attempts or with quit contemplation among US middle and high school students in any NYTS wave from 2011 to 2015.[20]

In existing studies of youth who had never smoked a cigarette at baseline, those who tried e-cigarettes were more likely to initiate cigarette smoking in the future.[2–6,9] In addition to smoking initiation among youth never-smokers, we demonstrate in the current study that e-cigarette use was also associated with progression to current established smoking among youth smoking experimenters.

In a study of California 10th grade students that included never smokers and current smokers at baseline, greater frequency of e-cigarette use at baseline was associated with subsequently greater levels of smoking frequency (days smoked in past month) and heaviness (cigarettes smoked per day) 6 months later.[21] Similarly, in a school-based study of adolescent never and current smokers in Canada, baseline past 30-day e-cigarette use was associated with initiation of daily smoking 1 year later.[4] In the results of a school-based study of baseline cigarette ever smokers in Hawaii, a statistically significant change at follow-up in smoking frequency (measured as numerical categories) between baseline e-cigarette ever and never users was not yielded.[2] However, in a school-based study of adolescents in the United Kingdom, ever use (versus never use) of e-cigarettes was associated with "escalation" to smoking sometimes or usually among baseline nonsmokers who had used cigarettes in the past.[5] Authors of that study reported an adjusted OR that was similar to the current study

(OR: 1.89; 95% CI: 0.82–4.33) but not statistically significant (P = .13) in a smaller sample (n = 318).[5]

The smoking outcomes evaluated in the current study represent intensity levels of clear clinical and public health concern. Although smoking as infrequently as 1 day in the past month in adolescence is predictive of adult smoking,[22] youth who reach higher levels of smoking are even more likely to continue to smoke.[12] Additionally, although more recent (past 30-day) e-cigarette use was a stronger predictor of future established smoking than former e-cigarette use in unadjusted models, this pattern did not necessarily persist in fully adjusted models. We suggest that any level of e-cigarette use among adolescent cigarette experimenters may be a meaningful risk indicator of smoking progression.

Several study advantages strengthened the conclusions that can be drawn from this research. The large, prospective, and nationally representative nature of the PATH Study enhanced generalizability and certainty regarding the temporal sequence between exposure and outcome. The PATH questionnaire was rigorously pilot tested and administered under a consistent protocol.[14] Furthermore, the magnitude of associations found in this study was largely consistent across different specifications of e-cigarette and cigarette use. Among other study aspects to consider, in-home administration of the PATH questionnaire could have led to differences in estimated tobacco use compared with school-based surveys. However, results of this analysis were qualitatively similar to previous work in which NYTS data was used.[13] As with all observational studies, residual confounding from unmeasured variables cannot be ruled out, although associations remained positive and at the threshold for statistical significance after adjustment for an extensive set of variables known to predict youth cigarette smoking.[23]

In July 2017, the US Food and Drug Administration announced a plan for tobacco and nicotine regulation that delayed federal e-cigarette regulation from 2018 until 2022.[24] However, local governments have taken regulatory action of e-cigarettes. For example, a 2017 San Francisco, California, ordinance prohibits the sale of flavored tobacco products, including e-cigarettes, with the intention of reducing the appeal of tobacco products to youth.[25] It is indicated in our results that among youth cigarette experimenters, those who have also used e-cigarettes are more likely to progress to current established smoking than those who tried cigarettes alone. As long as e-cigarettes remain attractive to youth, concern persists that these products contribute to greater combustible cigarette smoking among adolescents.

CONCLUSIONS

Among youth cigarette experimenters, using e-cigarettes was positively and independently associated with future onset of current established smoking, suggesting that e-cigarettes do not divert from, and may encourage, cigarette smoking in this population. In weighing the overall public health impact of e-cigarette availability, regulation, and use, the potential to increase combustible cigarette smoking by youth deserves special consideration.

ABBREVIATIONS

CI: confidence interval
e-cigarette: electronic cigarette
NYTS: National Youth Tobacco Survey
OR: odds ratio
PATH: Population Assessment of Tobacco and Health

PEDIATRICS (ISSN Numbers: Print, 0031-4005; Online, 1098-4275).

FINANCIAL DISCLOSURE: The authors have indicated they have no financial relationships relevant to this article to disclose.

FUNDING: Supported in part by the US National Cancer Institute (T32CA113710), the US Food and Drug Administration Center for Tobacco Products (P50CA180890), and the National Institute on Drug Abuse (R01DA043950). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the US Food and Drug Administration. The funding agencies played no role in the conduct of the research or the decision to submit the article for publication. Funded by the National Institutes of Health (NIH).

POTENTIAL CONFLICT OF INTEREST: The authors have indicated they have no potential conflicts of interest to disclose.

REFERENCES

1. Jamal A, Gentzke A, Hu SS, et al. Tobacco use among middle and high school students–United States, 2011-2016. *MMWR Morb Mortal Wkly Rep*. 2017;66(23):597–603
2. Wills TA, Knight R, Sargent JD, Gibbons FX, Pagano I, Williams RJ. Longitudinal study of e-cigarette use and onset of cigarette smoking among high school students in Hawaii. *Tob Control*. 2017;26(1):34–39
3. Barrington-Trimis JL, Urman R, Berhane K, et al. E-cigarettes and future cigarette use. *Pediatrics*. 2016;138(1):e20160379
4. Hammond D, Reid JL, Cole AG, Leatherdale ST. Electronic cigarette

use and smoking initiation among youth: a longitudinal cohort study. *CMAJ*. 2017;189(43):E1328–E1336

5. Conner M, Grogan S, Simms-Ellis R, et al. Do electronic cigarettes increase cigarette smoking in UK adolescents? Evidence from a 12-month prospective study [published online ahead of print August 17, 2017]. *Tob Control*. doi:10.1136/tobaccocontrol-2016-053539
6. Watkins SL, Glantz SA, Chaffee BW. Association of noncigarette tobacco product use with future cigarette smoking among youth in the Population Assessment of Tobacco and Health (PATH) study, 2013-2015 [published online ahead of print January 2, 2018]. *JAMA Pediatr*. doi:10.1001/jamapediatrics.2017.4173
7. Spindle TR, Hiler MM, Cooke ME, Eissenberg T, Kendler KS, Dick DM. Electronic cigarette use and uptake of cigarette smoking: a longitudinal examination of US college students. *Addict Behav*. 2017;67:66–72
8. Miech R, Patrick ME, O'Malley PM, Johnston LD. E-cigarette use as a predictor of cigarette smoking: results from a 1-year follow-up of a national sample of 12th grade students. *Tob Control*. 2017;26(e2):e106–e111
9. Soneji S, Barrington-Trimis JL, Wills TA, et al. Association between initial use of e-cigarettes and subsequent cigarette smoking among adolescents and young adults: a systematic review and meta-analysis. *JAMA Pediatr*. 2017;171(8):788–797
10. Schneider S, Diehl K. Vaping as a catalyst for smoking? An initial model on the initiation of electronic cigarette use and the transition to tobacco smoking among adolescents. *Nicotine Tob Res*. 2016;18(5):647–653
11. Etter JF. Gateway effects and electronic cigarettes [published online ahead of print August 7, 2017]. *Addiction*. doi:10.1111/add.13924
12. Sargent JD, Gabrielli J, Budney A, Soneji S, Wills TA. Adolescent smoking experimentation as a predictor of daily cigarette smoking. *Drug Alcohol Depend*. 2017;175:55–59
13. Dutra LM, Glantz SA. Electronic cigarettes and conventional cigarette use among US adolescents: a cross-sectional study. *JAMA Pediatr*. 2014;168(7):610–617
14. Hyland A, Ambrose BK, Conway KP, et al. Design and methods of the Population Assessment of Tobacco and Health (PATH) study. *Tob Control*. 2017;26(4):371–378
15. Evans N, Farkas A, Gilpin E, Berry C, Pierce JP. Influence of tobacco marketing and exposure to smokers on adolescent susceptibility to smoking. *J Natl Cancer Inst*. 1995;87(20):1538–1545
16. Stephenson MT, Hoyle RH, Palmgreen P, Slater MD. Brief measures of sensation seeking for screening and large-scale surveys. *Drug Alcohol Depend*. 2003;72(3):279–286
17. Judkins DR. Fay's method for variance estimation. *J Off Stat*. 1990;6(3):223–239
18. Rutten LJ, Blake KD, Agunwamba AA, et al. Use of e-cigarettes among current smokers: associations among reasons for use, quit intentions, and current tobacco use. *Nicotine Tob Res*. 2015;17(10):1228–1234
19. Kong G, Morean ME, Cavallo DA, Camenga DR, Krishnan-Sarin S. Reasons for electronic cigarette experimentation and discontinuation among adolescents and young adults. *Nicotine Tob Res*. 2015;17(7):847–854
20. Chaffee BW, Couch ET, Gansky SA. Trends in characteristics and multi-product use among adolescents who use electronic cigarettes, United States 2011-2015. *PLoS One*. 2017;12(5):e0177073
21. Leventhal AM, Stone MD, Andrabi N, et al. Association of e-cigarette vaping and progression to heavier patterns of cigarette smoking. *JAMA*. 2016;316(18):1918–1920
22. Saddleson ML, Kozlowski LT, Giovino GA, Homish GG, Mahoney MC, Goniewicz ML. Assessing 30-day quantity-frequency of U.S. adolescent cigarette smoking as a predictor of adult smoking 14 years later. *Drug Alcohol Depend*. 2016;162:92–98
23. Wellman RJ, Dugas EN, Dutczak H, et al. Predictors of the onset of cigarette smoking: a systematic review of longitudinal population-based studies in youth. *Am J Prev Med*. 2016;51(5):767–778
24. US Food and Drug Administration. FDA announces comprehensive regulatory plan to shift trajectory of tobacco-related disease, death. 2017. Available at: www.webcitation.org/6uf4uAFYK. Accessed November 1, 2017
25. Swan R. Group seeks referendum on flavored tobacco ban in S.F. *San Francisco Chronicle*. July 12, 2017. Available at: www.sfchronicle.com/politics/article/Group-seeks-referendum-on-flavored-tobacco-ban-in-11284771.php. Accessed July 12, 2017

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Journal of Adolescent Health 62 (2018) 539–547

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JOURNAL OF ADOLESCENT HEALTH

www.jahonline.org

Original article

The Association Between Smoking and Electronic Cigarette Use in a Cohort of Young People

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Article history: Received July 7, 2017; Accepted November 28, 2017
Keywords: Smoking; Electronic cigarettes; E-cigarettes; Young people; Youth; Adolescent; Longitudinal studies; Nicotine; Tobacco

A B S T R A C T

Purpose: Electronic cigarette (e-cigarette) use is associated with smoking initiation among young people; however, it is also possible that smoking is associated with e-cigarette initiation. This study explores these associations among young people in Great Britain.
Methods: A longitudinal survey of 1,152 11- to 18-year-olds was conducted with baseline in April 2016 and follow-up between August and October 2016. Logistic regression models and causal mediation analyses assessed whether (1) ever e-cigarette use and escalation were associated with smoking initiation (ever smoking at follow-up) among baseline never smokers (n = 923), and (2) ever smoking and escalation were associated with e-cigarette initiation (ever e-cigarette use at follow-up) among baseline never e-cigarette users (n = 1,020).
Results: At baseline, 19.8% were ever smokers and 11.4% were ever e-cigarette users. Respondents who were ever e-cigarette users (vs. never users, 53% vs. 8%, odds ratio [OR] = 11.89, 95% confidence interval [CI] = 3.56–39.72) and escalated their e-cigarette use (vs. did not, 41% vs. 8%, OR = 7.89, 95% CI = 3.06–20.38) were more likely to initiate smoking. Respondents who were ever smokers (vs. never smokers, 32% vs. 4%, OR = 3.54, 95% CI = 1.68–7.45) and escalated their smoking (vs. did not, 34% vs. 6%, OR = 5.79, 95% CI = 2.55–13.15) were more likely to initiate e-cigarette use. There was a direct effect of ever e-cigarette use on smoking initiation (OR = 1.34, 95% CI = 1.05–1.72), and ever smoking on e-cigarette initiation (OR = 1.08, 95% CI = 1.01–1.17); e-cigarette and smoking escalation, respectively, did not mediate these effects.

IMPLICATIONS AND CONTRIBUTION

This study employs a causal inference approach to provide further support for the association between ever e-cigarette use and smoking initiation, and additionally finds that ever smoking is associated with e-cigarette initiation, among young people.

Conflicts of Interest: Katherine East, Sara Hitchman, and Ann McNeill are members of the UK Centre for Tobacco and Alcohol Studies. Ioannis Bakolis is supported by the National Institute for Health Research (NIHR) Biomedical Research Centre at South London and Maudsley NHS Foundation Trust and by the NIHR Collaboration for Leadership in Applied Health Research and Care South London at King's College Hospital NHS Foundation Trust. Sarah Williams is an employee at Public Health England and was previously an employee at Action on Smoking and Health at the time this study was conducted. Hazel Cheeseman and Deborah Arnott are employees of Action on Smoking and Health, which receives funding from the British Heart Foundation, Cancer Research UK (CRUK), and the Department of Health. This study was funded by CRUK grant code A21559. CRUK was not involved in the study design, data collection, analysis or interpretation of the data, the write up of the manuscript, or decision to submit the article for publication. The views expressed are those of the author(s) and not necessarily those of Public Health England, CRUK, Action on Smoking and Health, the NHS, the NIHR or the Department of Health.

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https://doi.org/10.1016/j.jadohealth.2017.11.301

Conclusions: Among young people in Great Britain, ever e-cigarette use is associated with smoking initiation, and ever smoking is associated with e-cigarette initiation.

There are an estimated 2.9 million current adult electronic cigarette (e-cigarette) users in Great Britain [1]. Concerns have been expressed about the impact of e-cigarette use on cigarette smoking, particularly among young people [2–4]. There is some evidence that trial of e-cigarettes among young people aged 11–18 years in Great Britain is rising (from 3.7% in 2013 to 9.3% in 2016) [5]. However, regular (at least monthly) use among young people is low, and increases in regular use are mainly restricted to current smokers (from 20.2% in 2015 to 27.2% in 2016), with regular use by never smokers remaining rare (.6% in 2015 to .4% in 2016) [5].

Cross-sectional studies have found that young people who use e-cigarettes are more likely to smoke [6,7], intend to smoke [8,9], and be susceptible to smoking [10] than those who do not. On the other hand, among young people in Great Britain, ex- and current smokers are more likely to intend to use e-cigarettes than never smokers [11]. It is therefore difficult to determine whether there is any causality, and it is likely that there is an underlying factor driving both smoking and e-cigarette use.

Several longitudinal studies of U.S. youth have found baseline e-cigarette use is associated with smoking initiation [12–17], past six-month smoking [18], and past-month smoking [19] at follow-up. A meta-analysis of these studies has confirmed the strength and consistency of these associations [4], and the association between ever e-cigarette use and smoking initiation has since been replicated in England [20] and Scotland [21].

Although each of the above studies exploring the association between e-cigarette use and smoking control for a variety of factors associated with smoking, there remains the presence of extraneous variables, which may be related to both smoking and e-cigarette use. Furthermore, some researchers propose that certain psychosocial processes lead to vulnerability to any drug use [22,23]. One study [18] explored whether the association between smoking and e-cigarettes works both ways, and found that not only was use of e-cigarettes at baseline associated with past six-month smoking at follow-up, but also smoking at baseline was associated with past six-month e-cigarette use at follow-up. Furthermore, among young people in Argentina, current smoking was associated with e-cigarette initiation one and a half years later [24].

Despite the above research, the relative contributions of e-cigarette use to smoking initiation, and smoking to e-cigarette initiation, have not been formally assessed. All studies in this field with the exception of Wills and colleagues [15] have relied on standard regression models [12–14,16–21,24], which allow only limited conclusions to be drawn regarding the pathways between these products. Therefore, in this study, we have included causal mediation analyses [25] to investigate the causal influence of e-cigarette use on smoking initiation, and smoking on e-cigarette initiation.

This study is the first to our knowledge to explore the longitudinal association between (1) ever e-cigarette use and smoking initiation (ever smoking at follow-up) among baseline never smokers, and (2) ever smoking and e-cigarette initiation (ever e-cigarette use at follow-up) among baseline never e-cigarette users, among young people in Great Britain. We additionally explore whether escalation of each product between baseline and follow-up is associated with initiation of the alternative product, and employ causal mediation analyses for the identification of mediating factors [25] to investigate specific pathways between the two products.

Methods

Design

This study used data from the 2016 Action on Smoking and Health Great Britain Youth longitudinal survey. A non-probability quota sampling approach was adopted using Ipsos MORI's online panels to recruit respondents aged 11–18 years. Quotas were set in respect of age, gender, and Government Office Region (GOR) using data from Eurostat 2012 to ensure sample representativeness. Respondents were invited by email to participate in an online survey about smoking between April 6 and 20 with follow-up between August 5 and October 7, 2016. Up to eight email reminders were sent to maximize follow-up rates. Each wave took approximately 10 minutes to complete, and financial incentives were provided via a prize draw. Informed consent to take part in the surveys was provided either by the parents of those aged 11–15 years or by those individuals aged 16–18 years. Ethical approval for the analyses in this paper was not required as this study used secondary pre-existing data.

Ipsos MORI's online panel applicants consist of volunteers from the general public. These panel applicants are validated by a means of sophisticated vetting procedures using a variety of recruitment channels. Shortly after joining, panelists' survey-taking behavior is tested, with those most likely to make intentional or unintentional errors on future surveys deactivated. Subsequently, panelists' behavior is monitored and tracked across all surveys for quality reasons.

Sample

The baseline survey was completed by 2,916 respondents aged 11–18 years, of whom 1,469 (50%) successfully completed the follow-up survey. We excluded 317 respondents (22%) who had never heard of e-cigarettes and selected "don't know" or "prefer not to say" to some questions (see full breakdown in Figure 1). This left a final study sample of 1,152, of whom 923 (80%) were baseline never smokers and 1,020 (89%) were baseline never e-cigarette users (Figure 1).

Measures

Smoking and e-cigarette status. At baseline, respondents were classified as never smokers (never smoked, not even a puff) or ever smokers; at follow-up, respondents were classified as never smokers or initiated smoking (never smokers at baseline but ever

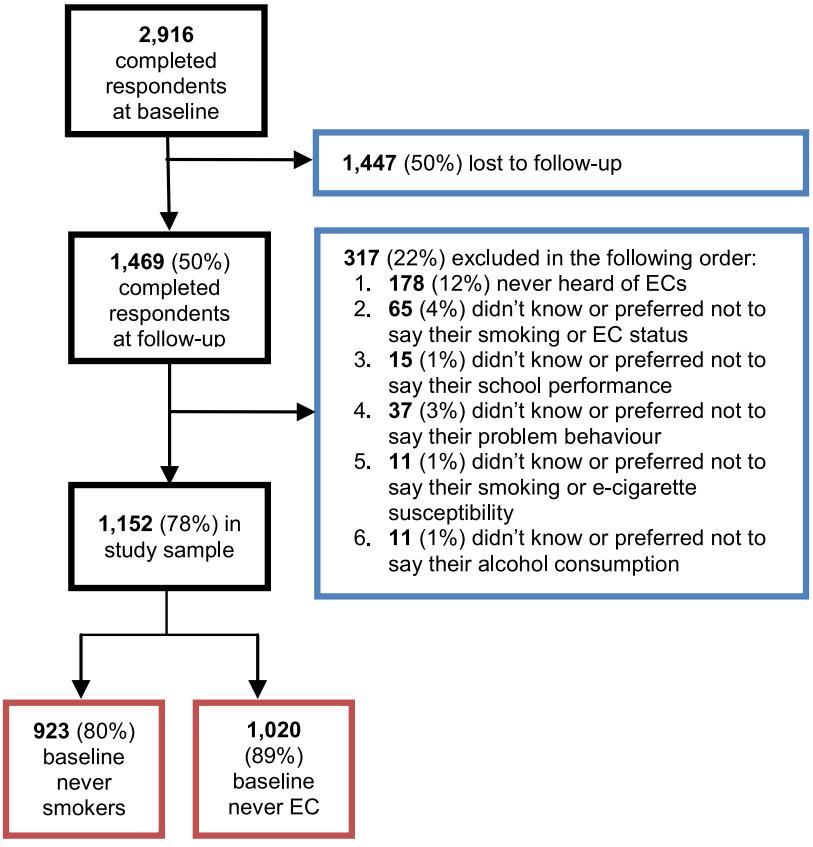

Figure 1. Flow diagram illustrating the respondent selection process. EC = electronic cigarette.

smokers at follow-up). At follow-up, respondents were further classified as having escalated smoking (increased their smoking between baseline and follow-up, e.g., escalating from never smoking to trying smoking, from smoking sometimes to smoking between one and six cigarettes a week) or not escalated smoking. Respondents were classified using the same procedure for e-cigarette use. Respondents who had never heard of e-cigarettes (n = 178), and those who responded with "Prefer not to say" or "Don't know" to the smoking or e-cigarette question at either baseline or follow-up (n = 65) were excluded from all analyses. Full item wording and response options are available in Table A1 (Supplementary Data).

Covariates (assessed at baseline only). Age (11–13, 14–15, 16–18), gender (male, female), school performance (1–4, below average to excellent), problem behavior (2–8, 8 = greater problem behavior), monthly alcohol use (yes, no), smoking susceptibility (susceptible, not susceptible) [26], e-cigarette susceptibility (susceptible, not susceptible—to mirror smoking susceptibility [26]), some friends smoke (yes, no, not applicable/don't know), some friends use e-cigarettes (yes, no, not applicable/don't know), at least one parent smokes (yes, no), at least one parent uses e-cigarettes (yes, no), sibling(s) smoke (yes, no, not applicable/don't know), sibling(s) use e-cigarettes (yes, no, not applicable/don't know), public approve of smoking (yes, no), and public approve of e-cigarettes (yes, no) [27]. For school performance, problem behavior, monthly alcohol use, and smoking and e-cigarette susceptibility, "Don't know" and "Prefer not to say" responses were excluded from all analyses. Covariates specific to smoking were selected based on the previous literature [12,15,18,26–28] and friend, parental, and sibling e-cigarette use and public approval of e-cigarettes were also included to mirror the similar smoking measures and to explore potential shared risk factors for each product. Full item wording, response options, and further details on coding for all covariates are available in Table A1 (Supplementary Data).

Statistical analysis

We used unadjusted logistic regressions to compare respondents lost to follow-up with those retained and included in the study sample. We then used chi-square tests to compare smoking and e-cigarette status at baseline and follow-up. We used unadjusted and adjusted logistic regressions to explore the associations between (1) ever e-cigarette use at baseline and e-cigarette escalation between baseline and follow-up with smoking initiation at follow-up among baseline never smokers (n = 923), and (2) ever smoking at baseline and smoking escalation between baseline and follow-up with e-cigarette initiation at follow-up among baseline never e-cigarette users (n = 1,020). In adjusted models, we adjusted for all covariates described in the Measures section.

To decompose the causal effect of e-cigarette use on smoking initiation, and smoking on e-cigarette initiation, we used causal mediation analyses using the parametric g-computation procedure [25]. Mediation analyses go beyond standard regression

models, which can estimate the associations between use of both products, by disentangling different pathways that could explain the effect of an exposure on an outcome. Furthermore, when a potential mediator is treated as confounder in standard regression models, spurious associations may arise. The most commonly used mediation analysis in epidemiology is based on the Baron and Kenny approach [29], in which the total effect of an exposure on an outcome, the effect of the exposure explained by a given set of mediators (indirect effect), and the effect of the exposure unexplained by those same mediators (direct effect) can be defined. This approach has four main problems as it (1) assumes no unmeasured confounding between mediator and outcome, (2) assumes no interactions between exposure and mediator on outcome, (3) does not extend to nonlinear models, and (4) assumes correctly specified models.

Causal mediation analysis has arisen from the causal inference literature [30] and addressed problems of the Baron and Kenny approach [29] under the potential outcomes framework, first by defining (using potential outcomes) precisely what is meant by direct and indirect effects, second by giving clear assumptions under which they can be identified, and third by generalizing the statistical methods available for carrying out such analyses to allow for nonlinearities, interactions, discrete outcomes, and semiparametric estimation [31]. We therefore use the parametric g-computation procedure under this framework as it can quantify reliable direct and indirect causal effects for binary variables, and produces narrow confidence intervals to allow for stronger conclusions to be made regarding observed associations [25,32]. The g-computation procedure is discussed in detail elsewhere [25,31,32], but primarily relies on the parametric modeling assumptions shared with logistic regression and, to infer causality, assumes no unmeasured confounding. It has been applied to survey data previously [33].

To assess the causal influence of e-cigarette use on smoking initiation, we specified a direct effect from ever e-cigarette use at baseline to smoking initiation at follow-up and an indirect effect acting through e-cigarette escalation between baseline and follow-up (mediator). We used the same approach to assess the causal influence of ever smoking on e-cigarette initiation at follow-up with smoking escalation between baseline and follow-up acting as a mediator. The causal diagrams for each model are shown in Figure 2. In the causal mediation analyses, all covariates described in the Measures section were specified as baseline confounders. The g-computation estimates were converted to odds ratios via exponentiation.

For attrition analysis and causal mediation analyses, we used unweighted data; for all other analyses, we used weighted data unless otherwise specified. Data were weighted according to age, gender, and GOR using data from the Eurostat 2012, and adjusted for attrition on age, gender, GOR, ever smoking, and ever e-cigarette use. Missing data were excluded listwise from all analyses (see Figure 1).

Results

Table 1 shows the characteristics of the study sample at baseline (n = 1,152) compared with respondents lost to follow-up and who would have otherwise been excluded (because of not having heard of e-cigarettes or selecting "don't know" or "prefer not to say" on key variables and covariates) (n = 1,225). Respondents were more likely to be lost to follow-up if they had ever smoked and ever used an e-cigarette, and also differed on all covariates included in the study except smoking susceptibility and having at least one parent who uses e-cigarettes.

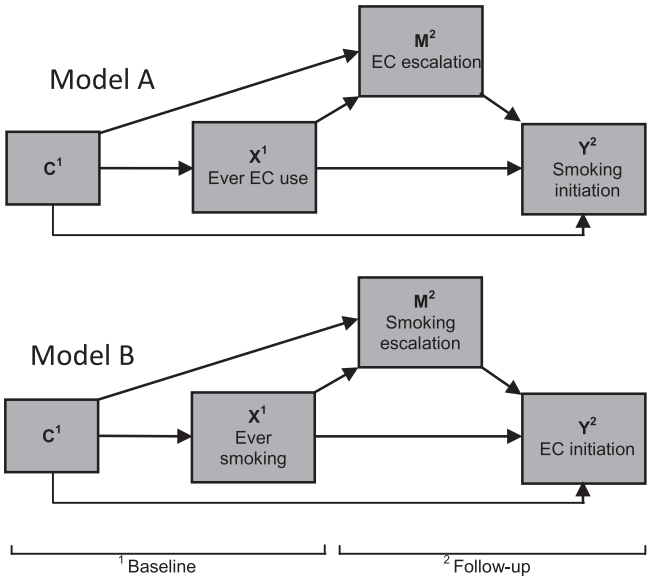

Figure 2. Conceptual causal diagrams for mediation and confounding. C = Covariate(s); X = Exposure; M = Mediator; Y = Outcome, EC = E-cigarette. Model A specifies baseline ever e-cigarette use as the exposure, e-cigarette escalation at follow-up as the mediator, and smoking initiation at follow-up as the outcome. Model B specifies baseline ever smoking as the exposure, smoking escalation at follow-up as the mediator, and e-cigarette initiation at follow-up as the outcome.

At baseline, 229 respondents (19.9%) had ever smoked (Table 1), and this increased to 301 (26.0%) at follow-up ($\chi^2 = 834.32$, $p < .001$). Of the 229 baseline ever smokers, 111 (48.5%) were also ever e-cigarette users; of the 923 baseline never smokers, 21 (2.3%) were ever e-cigarette users. At baseline, 132 respondents (11.5%) had ever used an e-cigarette (Table 1), increasing to 204 (17.6%) at follow-up ($\chi^2 = 761.74$, $p < .001$). Of the 132 baseline ever e-cigarette users, 111 (84.0%) were also ever smokers; of the 1,020 baseline never e-cigarette users, 118 (11.6%) were ever smokers. At baseline, only 56 (4.9%) respondents smoked monthly or more and 24 (2.1%) used an e-cigarette monthly or more.

Compared with baseline never e-cigarette users, ever e-cigarette users were more likely to initiate smoking at follow-up (Table 2). Furthermore, respondents who escalated e-cigarette use between baseline and follow-up were also more likely to initiate smoking at follow-up compared with those who did not (Table 2).

Compared with baseline never smokers, ever smokers were more likely to initiate e-cigarette use at follow-up (Table 3). Furthermore, respondents who escalated smoking between baseline and follow-up were also more likely to initiate e-cigarette use at follow-up compared with those who did not (Table 3).

Having some friends who use an e-cigarette reduced the likelihood of smoking initiation (Table 2) but increased the likelihood of e-cigarette initiation (Table 3). Being older, susceptible to smoking, and having at least one parent who smokes were associated with an increased likelihood of smoking initiation (Table 2). Monthly alcohol use and no perceived public approval of smoking were associated with an increased likelihood of e-cigarette initiation (Table 3).

Table 1
Respondent characteristics of the study sample at baseline (n = 1,152) and comparison with those lost to follow-up who would have otherwise been excluded (n = 1,225)

	Study sample (n = 1,152)	Lost to follow-up and excluded (n = 1,225)	OR (95% CI)
Ever smoked	229 (19.88)	382 (31.18)	**.55 (.45–.66)**
Ever used e-cigarettes	132 (11.46)	297 (24.24)	**.40 (.32–.51)**
Female	620 (53.82)	564 (46.04)	**1.37 (1.16–1.61)**
Age			
11–13	438 (38.02)	375 (30.61)	
14–15	338 (29.34)	263 (21.47)	1.10 (.89–1.36)
16–18	376 (32.64)	587 (47.92)	**.55 (.45–.66)**
School performance (1–4, 4 = excellent), mean (SD)	3.05 (.8)	2.97 (.8)	**1.11 (1.01–1.22)**
Problem behavior (2–8, 8 = high), mean (SD)	2.93 (1.2)	3.30 (1.4)	**.80 (.75–.86)**
Monthly alcohol use	269 (23.35)	407 (33.22)	**.61 (.51–.73)**
Susceptible to smoking	146 (12.67)	151 (12.33)	.86 (.67–1.11)
Susceptible to using e-cigarettes	264 (22.92)	330 (26.94)	**.63 (.52–.77)**
Some friends smoke			
No	371 (32.2)	279 (22.78)	
Yes	727 (63.11)	894 (72.98)	**.61 (.51–.73)**
DK/NA	54 (4.69)	52 (4.24)	.78 (.52–1.18)
Some friends use e-cigarettes			
No	684 (59.38)	526 (42.94)	
Yes	399 (34.64)	620 (50.61)	**.49 (.42–.59)**
DK/NA	69 (5.99)	79 (6.45)	**.67 (.48–.95)**
At least one parent smokes	343 (29.77)	413 (33.71)	**.83 (.70–.99)**
At least one parent uses e-cigarettes	182 (15.8)	221 (18.04)	.85 (.69–1.06)
Sibling(s) smokes			
No	918 (79.69)	935 (76.33)	
Yes	127 (11.02)	191 (15.59)	**.68 (.53–.86)**
NA/DK	107 (9.29)	99 (8.08)	1.10 (.83–1.47)
Sibling(s) use e-cigarettes			
No	992 (86.11)	1016 (82.94)	
Yes	54 (4.69)	119 (9.71)	**.46 (.33–.65)**
NA/DK	106 (9.20)	90 (7.35)	1.21 (.90–1.62)
Public approve of smoking	33 (2.86)	62 (5.06)	**.55 (.36–.85)**
Public approve of e-cigarettes	43 (3.73)	90 (7.35)	**.49 (.34–.71)**

All data are unweighted. Significant associations ($p < .05$) are highlighted in **bold.**
N (%) of the samples are reported unless otherwise stated.

In the causal mediation analysis (Figure 2, model A), baseline ever e-cigarette use had a direct causal effect on smoking initiation at follow-up (odds ratio [OR] = 1.34, 95% confidence interval [CI] = 1.05–1.72, $p = .018$), and there was a significant total causal effect of the model (OR = 1.35, 95% CI = 1.04–1.74, $p = .022$). However, there was no indirect effect of baseline ever e-cigarette use on smoking initiation at follow-up mediated by e-cigarette escalation between baseline and follow-up (OR = 1.00, 95% CI = .91–1.11, $p = .983$).

In the causal mediation analysis (Figure 2, model B), baseline ever smoking had a direct causal effect on e-cigarette initiation at follow-up (OR = 1.08, 95% CI = 1.01–1.17, $p = .034$), and there was a significant total causal effect of the model (OR = 1.11, 95% CI = 1.03–1.20, $p = .006$). However, there was no indirect effect of baseline ever smoking on e-cigarette initiation at follow-up mediated by smoking escalation between baseline and follow-up (OR = 1.03, 95% CI = .99–1.06, $p = .106$).

Discussion

This study was the first to explore the longitudinal association between e-cigarette use and smoking initiation, and smoking and e-cigarette initiation among young people in Great Britain, and to assess the relative contribution of these associations using a causal inference approach. In the logistic regression analyses, we found evidence for a prospective association between ever e-cigarette use and smoking initiation, and between ever smoking and e-cigarette initiation. We also found that escalation of each product (e-cigarettes and smoking) between baseline and follow-up was associated with initiation of the alternative product. The causal mediation analyses confirmed the direct effect of baseline ever e-cigarette use on smoking initiation, and baseline ever smoking on e-cigarette initiation, but found that e-cigarette and smoking escalation, respectively, did not mediate these effects.

This study provides insight into the impact of e-cigarette use on smoking and vice versa in young people; however, the findings must be considered in the light of some limitations. Attrition was high and respondents lost to follow-up differed substantially from those retained, potentially reducing generalizability to ever smokers, ever e-cigarette users, males, older respondents, and those with poorer school performance and greater problem behavior.

Although this study controlled for a variety of factors previously associated with smoking and e-cigarette use to enhance approximation of the models, there are still several factors that were not included that may contribute to the observed association between these products [28]. Examples may include curiosity, sensation seeking, liking, or disliking the effects of smoking/e-cigarettes, expectancies of smoking/e-cigarettes, mental ill health, and use of other drugs [28]. Furthermore, there are likely to be contributing factors that cannot be easily measured in surveys such as biological or genetic vulnerabilities, although drug use and parent's smoking and e-cigarette use may act as an indicator of these. Larger sample sizes are required to enable this substantial number of covariates to be assessed and meaningfully interpreted.

Table 2
Associations between smoking initiation at follow-up and e-cigarette use and all covariates, among baseline never smokers (n = 923)

	n (% initiated smoking)	Unadjusted		Adjusted model 1[a]		Adjusted model 2[b]	
		OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*
Baseline EC use							
Never	902 (8.2)	1.00		1.00		1.00	
Ever	21 (52.6)	**12.41 (4.53–33.99)**	**<.001**	**10.57 (3.33–33.50)**	**<.001**	**11.89 (3.56–39.72)**	**<.001**
Follow-up EC use							
No escalation	882 (8.1)	1.00		—	—	1.00	
Escalation	41 (41.0)	**7.94 (3.75–16.82)**	**<.001**	—	—	**7.89 (3.06–20.38)**	**<.001**
Age							
11–13	397 (4.4)	1.00		1.00		1.00	
14–15	270 (6.3)	1.45 (.71–2.97)	.312	1.22 (.54–2.73)	.636	1.35 (.58–3.15)	.485
16–18	256 (16.1)	**4.12 (2.19–7.76)**	**<.001**	**4.02 (1.72–9.40)**	**.001**	**4.98 (2.07–12.00)**	**<.001**
Gender							
Male	428 (10.8)	1.00		1.00		1.00	
Female	495 (8.5)	.77 (.46–1.30)	.331	.90 (.48–1.68)	.738	.91 (.47–1.76)	.786
School perf. (1–4, 4 = excellent)[c]	2.93 (.9)	.76 (.53–1.08)	.124	.91 (.64–1.29)	.596	.90 (.64–1.29)	.579
Problem beh. (2–8, 8 = high)[c]	3.05 (1.3)	**1.31 (1.03–1.66)**	**.028**	1.06 (.82–1.37)	.659	1.05 (.81–1.36)	.705
Monthly alcohol use							
No	790 (7.8)	1.00		1.00		1.00	
Yes	133 (18.1)	**2.61 (1.42–4.80)**	**.002**	1.64 (.82–3.30)	.165	1.32 (.61–2.86)	.480
Smoking susceptibility							
No	777 (7.9)	1.00		1.00		1.00	
Yes	146 (19.8)	**2.88 (1.57–5.29)**	**.001**	**2.38 (1.17–4.84)**	**.016**	**2.61 (1.23–5.52)**	**.012**
Some friends smoke							
No	355 (5.4)	1.00		1.00		1.00	
Yes	515 (12.9)	**2.60 (1.34–5.07)**	**.005**	1.48 (.66–3.34)	.341	1.28 (.57–2.87)	.555
NA/DK	53 (1.9)	.35 (.04–2.76)	.317	.30 (.04–2.43)	.258	.29 (.04–2.36)	.246
Some friends use EC							
No	598 (8.6)	1.00		1.00		1.00	
Yes	264 (11.0)	1.32 (.73–2.40)	.358	**.47 (.24–.93)**	**.029**	**.35 (.17–.75)**	**.007**
NA/DK	61 (15.1)	1.90 (.73–4.94)	.188	1.99 (.78–5.10)	.150	1.80 (.72–4.51)	.212
At least one parent smokes							
No	676 (6.8)	1.00		1.00		1.00	
Yes	247 (18.0)	**2.99 (1.72–5.20)**	**<.001**	**2.97 (1.62–5.44)**	**<.001**	**2.65 (1.37–5.12)**	**.004**
At least one parent uses EC							
No	802 (8.4)	1.00		1.00		1.00	
Yes	121 (18.8)	**2.54 (1.35–4.76)**	**.004**	1.47 (.70–3.07)	.304	1.33 (.65–2.73)	.437
Sibling(s) smoke							
No	761 (8.5)	1.00		1.00		1.00	
Yes	71 (20.8)	**2.83 (1.23–6.51)**	**.015**	.75 (.30–1.84)	.527	.84 (.33–2.16)	.723
NA/DK	91 (10.4)	1.25 (.56–2.82)	.584	1.65 (.56–4.92)	.365	1.94 (.66–5.69)	.226
Sibling(s) use EC							
No	810 (9.3)	1.00		1.00		1.00	
Yes	28 (24.3)	**3.13 (1.09–9.01)**	**.034**	2.16 (.54–8.58)	.274	1.59 (.35–7.27)	.551
NA/DK	85 (9.3)	1.00 (.41–2.41)	.998	.72 (.20–2.53)	.604	.67 (.19–2.41)	.543
Public approve of smoking							
No	903 (9.5)	1.00		1.00		1.00	
Yes	20 (20.5)	2.45 (.60–9.96)	.209	1.33 (.34–5.16)	.676	1.87 (.48–7.19)	.365
Public approve of ECs							
No	907 (9.7)	1.00		1.00		1.00	
Yes	16 (9.8)	1.00 (.20–4.99)	.997	.39 (.07–2.05)	.263	.40 (.08–1.92)	.252

Adjusted model 1 constant OR = .02 (95% CI = .00–.11) $p < .001$. Adjusted model 2 constant OR = .02 (95% CI = .00–.10), $p < .001$. N and % illustrate the number and percentage of individuals who initiated smoking at follow-up. All n use unweighted data, % and analyses use weighted data.
Significant associations ($p < .05$) are highlighted in **bold.**
beh. = behavior; EC = e-cigarette; perf. = performance.
[a] Adjusted model 1 is adjusted for all variables listed except follow-up EC use.
[b] Adjusted model 2 is adjusted for all variables listed.
[c] Mean(SD) reported, mean (SD) for never smoked at follow-up: school performance = 3.12 (.8), problem behavior = 2.71 (1.0).

Another important limitation is that this study uses the outcomes smoking initiation and e-cigarette initiation defined as progressing from never to ever use of each product. This is similar to some previous studies [12–16,21,24], yet the use of such broad measures has been criticized for providing limited evidence of progression to any significant smoking behavior [28,34]. However, because of low prevalence rates of monthly or more smoking (5%) and e-cigarette use (2%) in this study's sample, options for refining the measures were limited. Therefore, although the present study found an association between ever smoking and ever e-cigarette use, these cannot be generalized to current or regular use, and it cannot be determined whether e-cigarette experimentation leads to regular smoking. Such questions are critical in this area of research. Surveys with multiple waves across several years with larger sample sizes are needed to enable higher numbers of ever and current smokers and e-cigarette users, and further dissect the association between the two products.

Table 3
Associations between e-cigarette initiation at follow-up and smoking and all covariates, among baseline never e-cigarette users (n = 1,020)

	n (% initiated EC use)	Unadjusted		Adjusted model 1[a]		Adjusted model 2[b]	
		OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*
Baseline smoking							
Never	902 (4.1)	1.00		1.00		1.00	
Ever	118 (32.4)	**9.48 (5.36–16.76)**	**<.001**	**3.69 (1.88–7.23)**	**<.001**	**3.54 (1.68–7.45)**	**.001**
Follow-up smoking							
No escalation	932 (5.9)	1.00		—	—	1.00	
Escalation	88 (33.5)	**8.00 (4.36–14.69)**	**<.001**	—	—	**5.79 (2.55–13.15)**	**<.001**
Age							
11–13	413 (5.6)	1.00		1.00		1.00	
14–15	294 (6.1)	1.11 (.54–2.27)	.779	.65 (.29–1.43)	.285	.57 (.25–1.27)	.168
16–18	313 (12.5)	**2.41 (1.29–4.51)**	**.006**	.69 (.31–1.55)	.374	.48 (.19–1.18)	.109
Gender							
Male	468 (10.2)	1.00		1.00		1.00	
Female	552 (7.3)	.70 (.41–1.17)	.171	.77 (.41–1.43)	.404	.73 (.39–1.37)	.331
School perf. (1–4, 4 = excellent)[c]	2.67 (.9)	**.57 (.42–.78)**	**<.001**	.81 (.58–1.14)	.226	.79 (.55–1.12)	.183
Problem beh. (2–8, 8 = high)[c]	3.51 (1.4)	**1.62 (1.30–2.03)**	**<.001**	1.20 (.93–1.53)	.154	1.13 (.87–1.47)	.352
Monthly alcohol use							
No	824 (5.0)	1.00		1.00		1.00	
Yes	196 (20.6)	**4.93 (2.87–8.47)**	**<.001**	**2.66 (1.27–5.61)**	**.010**	**2.40 (1.08–5.33)**	**.032**
EC susceptibility							
No	756 (5.1)	1.00		1.00		1.00	
Yes	264 (18.9)	**4.39 (2.51–7.67)**	**<.001**	1.53 (.83–2.83)	.173	1.67 (.86–3.27)	.131
Some friends smoke							
No	363 (2.4)	1.00		1.00		1.00	
Yes	603 (12.3)	**5.58 (2.44–12.73)**	**<.001**	1.97 (.86–4.50)	.107	1.95 (.87–4.36)	.105
NA/DK	54 (5.5)	2.34 (.56–9.84)	.247	3.24 (.60–17.36)	.170	4.31 (.88–21.13)	.071
Some friends use EC							
No	660 (5.7)	1.00		1.00		1.00	
Yes	293 (15.9)	**3.14 (1.81–5.45)**	**<.001**	**2.69 (1.48–4.87)**	**.001**	**3.03 (1.63–5.64)**	**<.001**
NA/DK	67 (6.4)	1.15 (.31–4.19)	.835	1.10 (.20–6.14)	.915	.78 (.14–4.54)	.785
At least one parent smokes							
No	733 (6.6)	1.00		1.00		1.00	
Yes	287 (14.9)	**2.47 (1.45–4.23)**	**.001**	1.88 (.91–3.91)	.090	1.45 (.61–3.46)	.405
At least one parent uses EC							
No	884 (7.6)	1.00		1.00		1.00	
Yes	136 (17.3)	**2.54 (1.38–4.67)**	**.003**	2.34 (1.00–5.47)	.051	2.1 (.87–5.07)	.097
Sibling(s) smoke							
No	830 (7.4)	1.00		1.00		1.00	
Yes	94 (24.0)	**3.94 (2.00–7.75)**	**<.001**	1.49 (.66–3.36)	.332	1.64 (.69–3.91)	.266
NA/DK	96 (3.9)	.51 (.16–1.61)	.251	.36 (.06–2.11)	.258	.27 (.04–1.93)	.193
Sibling(s) use EC							
No	899 (8.3)	1.00		1.00		1.00	
Yes	31 (29.9)	**4.69 (1.50–14.66)**	**.008**	1.46 (.39–5.43)	.576	.92 (.28–3.09)	.895
NA/DK	90 (5.6)	.66 (.23–1.83)	.420	1.03 (.21–5.11)	.969	1.10 (.19–6.27)	.917
Public approve of smoking							
No	1000 (9.0)	1.00		1.00		1.00	
Yes	20 (2.8)	.29 (.04–2.22)	.233	**.09 (.01–.88)**	**.038**	.15 (.02–1.22)	.076
Public approve of ECs							
No	995 (8.5)	1.00		1.00		1.00	
Yes	25 (20.9)	2.84 (.95–8.50)	.061	.99 (.31–3.15)	.987	1.32 (.34–5.15)	.689

Adjusted model 1 constant OR = .02 (95% CI = .00–.07) $p < .001$. Adjusted model 2 constant OR = .02 (95% CI = .00–.10), $p < .001$. N and % illustrate the number and percentage of individuals who initiated EC use at follow-up. All n use unweighted data, % and analyses use weighted data.
Significant associations ($p < .05$) are highlighted in **bold.**
beh. = behavior; EC = e-cigarette; perf. = performance.
[a] Adjusted model 1 is adjusted for all variables listed except follow-up smoking.
[b] Adjusted model 2 is adjusted for all variables listed.
[c] Mean (SD) reported, mean (SD) for never used EC at follow-up: school performance = 3.08 (.8), problem behavior = 2.77 (1.0).

Despite the above limitations, this study has several strengths. It was the first to explicitly explore the association not only between e-cigarette use at baseline and smoking initiation at follow-up but additionally smoking at baseline and e-cigarette initiation at follow-up. Moreover, a novel statistical approach (causal mediation analysis [25]) was used to explore whether the association between baseline ever e-cigarette use and smoking initiation at follow-up was mediated by escalation of e-cigarette use between survey waves; the same procedure was also used to explore further the association between smoking and e-cigarette initiation. To our knowledge this has not been done previously. Finally, the sample was drawn from the general population in Great Britain using a quota sampling approach to enhance representativeness.

The rate of ever smoking in this study was 19.9% at baseline, which is lower than other findings in Great Britain in 2016 [5], but could be because of those lost at follow-up being more likely to smoke. The rate of ever e-cigarette use (11.5% at baseline) and

findings that ever e-cigarette use was largely confined to those who had ever smoked, with a low proportion of never smokers having ever used e-cigarettes, was consistent with other findings in Great Britain [5,35]. Furthermore, only 4% of never smokers initiated e-cigarette use (vs. 32% of ever smokers). This suggests that e-cigarettes are attracting few who have never smoked. Furthermore, monthly or more smoking and e-cigarette use was low, at 5% and 2%, respectively.

In the logistic regression analyses, e-cigarette escalation between baseline and follow-up was associated with smoking initiation, even when controlling for ever e-cigarette use; likewise, smoking escalation was associated with e-cigarette initiation when controlling for ever smoking. This represents a novel contribution to the literature, and further suggests the need for multiwave surveys to explore dynamic changes in use of both products over time. Despite this, the causal mediation analyses, which as discussed allow for stronger conclusions to be made regarding observed observations, suggest that it is primarily ever use of that product that contributes to initiation of the alternative product.

Our findings are consistent with previous studies that found a prospective association between e-cigarette use at baseline and smoking at follow-up [4,12–21], and also with those who found a prospective association between smoking at baseline and e-cigarette use at follow-up [18,24]. There are several possible reasons for the strong and reliable association between e-cigarettes and smoking in young people [18,28,36]. One interpretation is that e-cigarettes act as a "gateway" to smoking [3,37]; however, this has been contested [28,36], and our findings suggest that the association between e-cigarette initiation and smoking initiation may work both ways. Certain psychological processes ("common liabilities") may lead to vulnerability of any drug use [22,23]. Specifically, young people who exhibit curiosity, rebelliousness, and sensation-seeking may be more likely to experiment with both smoking and e-cigarettes. Future research should explore potential common liabilities pertaining to experimentation of both products, some of which were included in this study and others are proposed above.

Despite potential common liabilities and our findings that e-cigarette use is associated with smoking and vice versa, there are several important differences to consider between these products and the contexts in which they may be used. Among young people, e-cigarettes, compared with conventional cigarettes, have been described as more accessible and convenient [38,39], have a greater capacity for continual novelty in terms of flavors and devices [39], and are perceived as less harmful in the UK [5,39]. On the contrary, smoking is highly stigmatized in some societal groups [40]. Indeed, some have reported that e-cigarettes appeal to those who do not want to smoke but want to try the experience of "smoking" [38,39].

Interestingly, friend's e-cigarette use increased the likelihood of e-cigarette initiation but reduced the likelihood of smoking initiation in adjusted models. This first association is unsurprising given the important role of peer influence on behavior. However, the protective effect of friend's e-cigarette use on smoking initiation warrants further investigation.

In conclusion, this study provides further support for the association between ever e-cigarette use and smoking initiation, and additionally finds that ever smoking is associated with e-cigarette initiation, among young people. Better understanding of these associations will aid policy makers with their efforts to develop an appropriate regulatory framework for both tobacco products and e-cigarettes.

Funding Sources

This work was funded by Cancer Research UK grant code A21559. Thanks are also given to the UK Public Health Research Consortium (grant number PHPEHF50/13) for funding the development of some of the covariates included in this study.

Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jadohealth.2017.11.301.

References

[1] Action on Smoking and Health. Use of e-cigarettes (vapourisers) among adults in Great Britain. 2017.
[2] Fairchild AL, Bayer R, Colgrove J. The renormalization of smoking? E-cigarettes and the tobacco "endgame." N Engl J Med 2014;370:293–5. doi:10.1056/NEJMp1313940.
[3] Kmietowicz Z. E-cigarettes are "gateway devices" for smoking among young people, say researchers. BMJ 2014;348:g2034. doi:10.1136/bmj.g2034.
[4] Soneji S, Barrington-Trimis JL, Wills TA, et al. Association between initial use of e-cigarettes and subsequent cigarette smoking among adolescents and young adults: A systematic review and meta-analysis. JAMA Pediatr 2017;171:788–97. doi:10.1001/jamapediatrics.2017.1488.
[5] Eastwood B, East K, Brose LS, et al. Electronic cigarette use in young people in Great Britain 2015–2016. Public Health 2017;149:45–8. doi:10.1016/j.puhe.2017.03.019.
[6] Dutra LM, Glantz SA. Electronic cigarettes and conventional cigarette use among U.S. adolescents: A cross-sectional study. JAMA Pediatr 2014;168:610–7. doi:10.1001/jamapediatrics.2013.5488.
[7] Barrington-Trimis JL, Berhane K, Unger JB, et al. Psychosocial factors associated with adolescent electronic cigarette and cigarette use. Pediatrics 2015;136:308–17. doi:10.1542/peds.2015-0639.
[8] Bunnell RE, Agaku IT, Arrazola RA, et al. Intentions to smoke cigarettes among never-smoking US middle and high school electronic cigarette users: National Youth Tobacco Survey, 2011–2013. Nicotine Tob Res 2015;17:228–35. doi:10.1093/ntr/ntu166.
[9] Moore GF, Littlecott HJ, Moore L, et al. E-cigarette use and intentions to smoke among 10-11-year-old never-smokers in Wales. Tob Control 2016;25:147–52. doi:10.1136/tobaccocontrol-2014-052011.
[10] Trinidad DR, Pierce JP, Sargent JD, et al. Susceptibility to tobacco product use among youth in wave 1 of the population Assessment of tobacco and health (PATH) study. Prev Med 2017;101:8–14. doi:10.1016/j.ypmed.2017.05.010.
[11] Eastwood B, Dockrell M, Arnott D, et al. Electronic cigarette use in young people in Great Britain 2013–2014. Public Health 2015;129:1150–6. doi:10.1016/j.puhe.2015.07.009.
[12] Barrington-Trimis JL, Urman R, Berhane K, et al. E-cigarettes and future cigarette use. Pediatrics 2016;138:doi:10.1542/peds.2016-0379.
[13] Miech R, Patrick ME, O'Malley PM, et al. E-cigarette use as a predictor of cigarette smoking: Results from a 1-year follow-up of a national sample of 12th grade students. Tob Control 2017;1–6. doi:10.1136/tobaccocontrol-2016-053291.
[14] Primack BA, Soneji S, Stoolmiller M, et al. Progression to traditional cigarette smoking after electronic cigarette use among US adolescents and young adults. JAMA Pediatr 2015;169:1018–23. doi:10.1001/jamapediatrics.2015.1742.
[15] Wills TA, Gibbons FX, Sargent JD, et al. How is the effect of adolescent e-cigarette use on smoking onset mediated: A longitudinal analysis. Psychol Addict Behav 2016;30:876–86. doi:10.1037/adb0000213.
[16] Wills TA, Knight R, Sargent JD, et al. Longitudinal study of e-cigarette use and onset of cigarette smoking among high school students in Hawaii. Tob Control 2016;26:34–9. doi:10.1136/tobaccocontrol-2015-052705.
[17] Spindle TR, Hiler MM, Cooke ME, et al. Electronic cigarette use and uptake of cigarette smoking: A longitudinal examination of U.S. college students. Addict Behav 2017;67:66–72. doi:10.1016/j.addbeh.2016.12.009.
[18] Leventhal AM, Strong DR, Kirkpatrick MG, et al. Association of electronic cigarette use with initiation of combustible tobacco product smoking in early adolescence. JAMA 2015;314:700–7. doi:10.1001/jama.2015.8950.
[19] Unger JB, Soto DW, Leventhal A. E-cigarette use and subsequent cigarette and marijuana use among Hispanic young adults. Drug Alcohol Depend 2016;163:261–4. doi:10.1016/j.drugalcdep.2016.04.027.
[20] Conner M, Grogan S, Simms-Ellis R, et al. Do electronic cigarettes increase cigarette smoking in UK adolescents? Evidence from a 12-month prospective study. Tob Control 2017;doi:10.1136/tobaccocontrol-2016-053539. Published online first: 17 August 2017.

[21] Best C, Haseen F, Currie D, et al. Relationship between trying an electronic cigarette and subsequent cigarette experimentation in Scottish adolescents: A cohort study. Tob Control 2017;doi:10.1136/tobaccocontrol-2017-053691. Published Online First: 22 July 2017.
[22] Van Leeuwen AP, Verhulst FC, Reijneveld SA, et al. Can the gateway hypothesis, the common liability model and/or, the route of administration model predict initiation of cannabis use during adolescence? A survival analysis—the TRAILS study. J Adolesc Health 2011;48:73–8.
[23] Vanyukov MM, Tarter RE, Kirillova GP, et al. Common liability to addiction and "gateway hypothesis": Theoretical, empirical and evolutionary perspective. Drug Alcohol Depend 2012;123(S1):S3–17. doi:10.1016/j.drugalcdep.2011.12.018.
[24] Morello P, Perez A, Peña L, et al. Prevalence and predictors of e-cigarette trial among adolescents in Argentina. Tob Prev Cess 2016;2:doi:10.18332/tpc/66950.
[25] Daniel RM, De Stavola BL, Cousens SN. gformula: Estimating causal effects in the presence of time-varying confounding or mediation using the g-computation formula. Stata J 2011;11:479–517.
[26] Pierce JP, Choi WS, Gilpin EA, et al. Validation of susceptibility as a predictor of which adolescents take up smoking in the United States. Health Psychol 1996;15:355–61. doi:10.1037/0278-6133.15.5.355.
[27] East K, Hitchman SC, Stead M, et al. The development of tools to measure norms towards smoking, nicotine use, and the tobacco industry. 2017.
[28] Kozlowski LT, Warner KE. Adolescents and e-cigarettes: Objects of concern may appear larger than they are. Drug Alcohol Depend 2017;174:209–14. doi:10.1016/j.drugalcdep.2017.01.001.
[29] Baron RM, Kenny DA. The moderator-mediator variable distinction in social psychological research—conceptual, strategic, and statistical considerations. J Pers Soc Psychol 1986;51:1173–82.
[30] Vanderweele TJ. Explanation in causal inference: Methods for mediation and interaction. Oxford University Press; 2015.
[31] De Stavola BL, Daniel RM, Ploubidis GB, et al. Mediation analysis with intermediate confounding: Structural equation modeling viewed through the causal inference lens. Am J Epidemiol 2015;181:64–80. doi:10.1093/aje/kwu239.
[32] Wang A, Arah OA. G-computation demonstration in causal mediation analysis. Eur J Epidemiol 2015;30:1119–27. doi:10.1007/s10654-015-0100-z.
[33] Zhang YT, Laraia BA, Mujahid MS, et al. Does food vendor density mediate the association between neighborhood deprivation and BMI? A G-computation mediation analysis. Epidemiol 2015;26:344. doi:10.1097/EDE.0000000000000271.
[34] Rigotti NA. E-cigarette use and subsequent tobacco use by adolescents: New evidence about a potential risk of e-cigarettes. JAMA 2015;314:673–4. doi:10.1001/jama.2015.8382.
[35] Bauld L, MacKintosh AM, Eastwood B, et al. Young people's use of e-cigarettes across the United Kingdom: Findings from five surveys 2015–2017. Int J Environ Res Public Health 2017;14:doi:10.3390/ijerph14090973.
[36] McNeill A, Brose LS, Calder R, et al. E-cigarettes: an evidence update: A report commissioned by Public Health England. 2015.
[37] Bell K, Keane H. All gates lead to smoking: The "gateway theory", e-cigarettes and the remaking of nicotine. Soc Sci Med 2014;119:45–52. doi:10.1016/j.socscimed.2014.08.016.
[38] Choi K, Fabian L, Mottey N, et al. Young adults' favorable perceptions of snus, dissolvable tobacco products, and electronic cigarettes: Findings from a Focus Group Study. Am J Public Health 2012;102:doi:10.2105/AJPH.2011.300525.
[39] Hilton S, Weishaar H, Sweeting H, et al. E-cigarettes, a safer alternative for teenagers? A UK focus group study of teenagers' views. BMJ Open 2016;6:e013271. doi:10.1136/bmjopen-2016-013271.
[40] McCool J, Hoek J, Edwards R, et al. Crossing the smoking divide for young adults: Expressions of stigma and identity among smokers and nonsmokers. Nicotine Tob Res 2013;15:552–6. doi:10.1093/ntr/nts136.

Regulatory Toxicology and Pharmacology 95 (2018) 8–16

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

In-silico study of toxicokinetics and disease association of chemicals present in smokeless tobacco products

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ARTICLE INFO

Keywords:
Regulation
Toxicity
Smokeless tobacco
Toxicokinetics
Pathway
Carcinogen
Chemical compounds
ADMET

ABSTRACT

Smokeless tobacco (SLT) products are consumed by millions of people in over 130 countries around the world. Consumption of SLT has been estimated to cause a number of diseases accounting to more than 0.65 million deaths per year. There is sufficient epidemiological evidence on the association of SLT products with nicotine addiction, cancers of oral cavity and digestive systems but there is a lack of understanding of the role of toxic chemicals in these diseases. We provide the first comprehensive *in-silico* analysis of chemical compounds present in different SLT products used worldwide. Many of these compounds are found to have good absorption, solubility and permeability along with mutagenic and toxic properties. They are also found to target more than 350 human proteins involved in a plethora of human biological processes and pathways. Along with all the previously known diseases, the present study has identified the association of compounds of SLT products with a number of unknown diseases like neurodegenerative, immune and cardiac diseases (Left ventricular non compaction, dilated cardiomyopathy etc). These findings indicate far-reaching impact of SLT products on human health than already known which needs further validations using epidemiological, in-vitro and in-vivo methodologies. Thus, this study will provide one stop information for the policy makers in development of regulatory policies on toxic contents of SLT products.

1. Introduction

Smokeless tobacco (SLT) products or non-combustible tobacco products can be chewed, snuffed orally, nasally, applied over gums and teeth, gargled or drunk. Over 350 million SLT users live in more than 133 countries of the world and over 0.65 million deaths annually are caused due to SLT use (Sinha et al., 2018). There is sufficient evidence on the association of SLT products with nicotine addiction, cancers of oral cavity and digestive systems, coronary heart disease, diseases of the nervous system (stroke) and aberrant reproductive outcomes (still birth, low birth weight babies) (Boffetta and Straif, 2009; Gupta and Johnson, 2014; Piano et al., 2010; Siddiqi et al., 2015; Sinha et al., 2016, 2018).

SLT products are known to contain diverse classes of chemical compounds namely alkaloids, Tobacco-specific N-nitrosamines (TSNAs), N-nitrosamino acids, volatile N-nitrosamines, poly aromatic hydrocarbons (PAHs) and volatile aldehydes etc (Borgerding et al., 2012). These exist in varied proportions according to the product, geographical location, processing and type of tobacco used for making the product. In commercially used tobacco plant, nicotine is the predominant alkaloid representing 90–95% of the total alkaloid content (Siminszky et al., 2005). Absorption of nicotine is mainly dependent on the levels of the unprotonated or free nicotine that is influenced by the level of pH. The International Agency for Research on Cancer (IARC) has identified carcinogenic chemical compounds present in tobacco products and classified them on the basis of levels of carcinogenicity (Thun and Jemal, 2003). The most potent Group 1 carcinogens include some TSNAs, (N-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1 (3-pyridyl)-1 butanone (NNK)) and Poly Aromatics Hydrocarbons (benzo[a]pyrene (B[a]P) (Supplementary Table I).

Several additives like areca nut added to SLT products to enhance their flavor, taste and aroma. Additives like areca nut contain known carcinogens and potentiates carcinogenesis (Shah et al., 2012; Sharan et al., 2012). However, the functional analysis of the compounds found

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https://doi.org/10.1016/j.yrtph.2018.03.002
Received 6 October 2017; Received in revised form 11 January 2018; Accepted 1 March 2018
Available online 02 March 2018
0273-2300/

in these additives is very limited.

The levels of the TSNA per unit dose in SLT products have been reported to be higher than those of cigarette smoke (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 2007). Biomonitoring of TSNAs and their metaboliteshave shown presence of NNN, NNK, N′-nitrosoanabasine (NAB) and N′-nitrosoanatabine (NAT) in saliva and urine of users of betel quid with tobacco, toombak, khaini and snuff dippers. The toxicokinetics which include absorption, distribution, metabolism, excretion and toxicity of a few TSNAs has been studied (Adams et al., 1985, 1984; Pool-Zobel et al., 1991). The distribution and metabolism of a few TSNAs have also been studied but such studies for other classes of compounds of SLT products are missing. Functional annotation of the targets of the chemical compounds of SLT products has not been performed.

Although there is sufficient evidence on the association of SLT products with nicotine addiction and different cancers, not much emphasis has been given on their regulation. SLT products contain 39 chemicals which are classified as Group 1 and Group 2A&B carcinogens by IARC. Although a lot of studies are available on TSNAs, there is a lack of research on other chemicals and their associated health effects. This study presents for the first time a comprehensive analysis of toxicokinetic properties and protein targets of compounds of SLT products to identify their potential role is diseases associated with SLT consumption. Experimental validation of the compounds, identified as potentially disease causing, may help in further establishing role of SLT chemicals in human diseases and developing appropriate regulatory policies by the policy makers. Effective regulation of tobacco products will be the first milestone to achieve a bigger objective of reducing the danger of SLT products.

2. Materials and methods

2.1. Developing comprehensive list of chemical compounds present in SLT products

The information about chemical composition of different SLT products was extracted by extensively searching peer-reviewed literature resources like PubMed, published books, IARC monographs and reports using various combinations of keywords (Fig. 1) (Borgerding et al., 2012; Brunnemann et al., 1985; Idris et al., 1991; Klus et al., 2014; National Cancer Institute and Centers for Disease Control and Prevention and Bethesda, 2014; Rickert et al., 2009; Stanfill et al., 2015; Stepanov et al., 2010, 2008; Tricker and Preussmann, 1988; U.S. Department of Health and Human Services and National Institutes of Health, 2014; Verma et al., 2010).

2.2. Identification of similar compounds

Similarity search algorithms were used to identify compounds similar to the compounds of SLT products ChEMBL database release 21 (Gaulton et al., 2012). Similarity search required input of three dimensional structures of compounds. For generating the three dimensional structures, the two dimensional structures were first drawn using open source drawing tool JChemPaint (Krause et al., 2000) and were further converted to three dimensional using Open Babel (O'Boyle et al., 2011).

Similarity search between chemical compounds of SLT products and ChEMBL database release 21 was performed using Open Babel-2.3.2. Tanimoto coefficient was used as the primary cutoff to filter similar compounds (Jaccard, 1901; Tanimoto, 1957). A stringent cutoff of Tanimoto coefficient was used in the study to avoid spurious similarities. It may restrict the search space, however will also restrict the false hits. This screened list of similar compounds from ChEMBL was further compared with the respective compounds of SLT products on the basis of their molecular weight using *in-house* Perl scripts.

2.3. Identification of protein targets and functional annotation

The information on protein targets interacting with compounds found in SLT products was extracted from the ChEMBL database using results of similarity search. The targets identified from ChEMBL are categorized on the basis of confidence score which represents the confidence that the target assigned is the correct target for the particular assay. Out of scores ranging from 0 to 9, selected targets were with confidence scores 8 or 9 representing experimentally validated homologous single protein and direct single protein targets respectively.

Functional mapping of the identified targets to pathway and Gene Ontologies was performed using PANTHER 11.1 (Thomas et al., 2003). *In-house* Perl scripts were used for mapping and further analysis. The diseases associated with the protein targets were extracted from Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2017; Kanehisa and Goto, 2000).

2.4. Toxicokinetic analysis

The toxicokinetic analysis of compounds present in SLT products was performed using Discovery Studio (DS) Version 4.0 and pkCSM server (Pires et al., 2015). Absorption, Solubility, Blood Brain Barrier (BBB), Plasma Protein Binding, CYP2D6 inhibition, hepatotoxicity were calculated using ADMET module and toxicity profile having carcinogenicity and Ames Mutagenicity of the compounds were predicted using the TOPKAT (Toxicity Prediction by Komputer Assisted Technology) module of Discovery Studio version 4.0. Few other important parameters such as Caco-2 permeability, Vdss and CNS permeability were calculated by pkCSM web server (Supplementary Table II).

Permeability prediction was carried out by pkCSM server on the model built on Caco-2 permeability data. The Caco-2 cell line is composed of human epithelial colorectal adenocarcinoma cells. This cell line is used as an in vitro model to predict the absorption of orally administered compounds. A compound is considered to have a high Caco-2 permeability provided the apparent permeability coefficient (Papp) $> 8 \times 10^{-6}$ cm/s. The cut off for high Caco-2 permeability in pkCSM predictive model is value of logarithm of Papp (log Papp) > 0.90 (Pires et al., 2015).

Blood brain barrier model predicts the ability of chemical compounds to penetrate the blood brain barrier. This model was derived from a quantitative linear regression model using over 800 compounds that are known to enter the CNS after oral administration (Ponnan et al., 2013). Compounds penetrating the Central Nervous System were also identified by calculating the parameter CNS permeability, which is defined as product of blood-brain permeability and surface area (logPS) (Pires et al., 2015). Compounds predicted with a logPS > -2 by pkCSM server are considered to penetrate the Central Nervous System. Parameter Vdss (Volume of distribution) was used to check the distribution of compounds of SLT products. Parameter Vdss is the theoretical volume of a chemical compound needed to maintain the same concentration as in blood plasma (Pires et al., 2015). Higher the value of Vdss, greater amount of chemical is distributed in tissue as compared to plasma. Vdss is considered to be high if the value is greater than 2.81 L/kg (log Vdss > 0.45).

Plasma protein binding (PPB) is another parameter which defines the degree to which a chemical binds plasma proteins in blood. Unbound chemicals to plasma proteins are more efficiently available to diffusion across cell membrane. 45 compounds of SLT products were predicted to be unbound to plasma proteins. CYP2D6 protein plays an important role in metabolism of xenobiotics. In order to understand the side effect of a chemical in humans, it is important to measure its ability to inhibit cytochrome P450. Our analysis shows that 18 compounds were predicted to be inhibitors of CYP2D6.

Toxicity was predicted by AMES mutagenicity model of Discovery Studio which tests a chemical for its mutagenic properties. TOPKAT

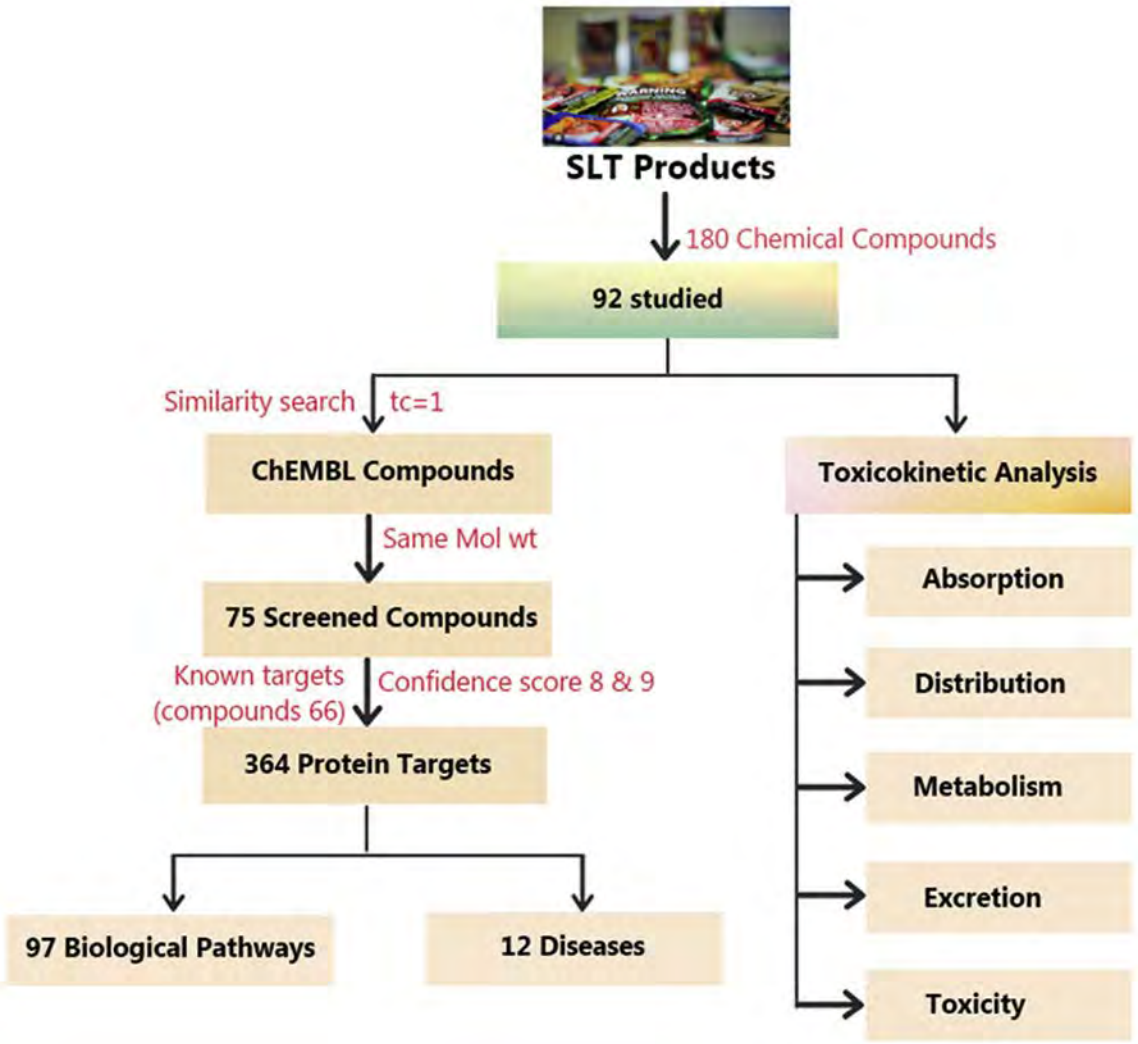

Fig. 1. Flowchart depicting the workflow followed.

mutagenicity predictor of Discovery Studio identifies structural features common to the submitted structure. Those compounds whose computed probability of mutagenicity is greater than 0.7 are considered to be mutagen (Snyder et al., 2004).

These studies are solely based on structures of the compounds of SLT which were uploaded as SMILES strings or 3D structures in the software. ADMET descriptor values of Discovery Studio and pkCSM server and their corresponding interpretations are given in Supplementary Table II.

2.5. Chemical-target-disease interaction networks

The interaction networks between compounds found in SLT products and protein targets were constructed using Cytoscape version 3.4.0 (Shannon et al., 2003). The network was analyzed using the plug-in Network Analyser. The unique interactions between the compounds of SLT products and protein targets were used as edges and the nodes were color coded on the basis of the feature specific information. All the compounds were given red color and the protein targets were provided the color green.

3. Results

3.1. Chemical composition of compounds

We compiled a list of 180 chemical compounds belonging to 22 chemical classes from different SLT products reported in peer-reviewed literature (Supplementary Table I). Of these 180 compounds, 7 compounds were classified as Group 1 carcinogens to humans according to IARC monographs (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 2004, 2007, 2012) (Supplementary Table I). Out of the 180 identified compounds, common compounds of classes such as heavy metals and inorganic compounds, radioactive elements, sweeteners, humectants, phenols in betel leaf, alkaline agents, essential oils, phytosterols, pesticide residues, mycotoxins, and stimulants were excluded. Present study includes 92 compounds based on the availability of chemical structures for further calculation.

3.2. Identification of protein targets of compounds found in SLT products

Similarity search algorithms were utilized to identify compounds similar to 92compounds of SLT products.

Of the total 15,83,897 ChEMBL compounds, 421 compounds were found to be similar to 88 compounds of SLT products using Tanimoto coefficient cutoff (tc = 1). On comparing the molecular weight, we found that 75 compounds of SLT products have same molecular weight

Table 1
Distribution of protein targets in Panther protein classes.

S. No.	Protein class	No of proteins
1	Receptor	74
2	Transferase	63
3	Hydrolase	45
4	Nucleic acid binding	40
5	Oxidoreductase	37
6	Transcription factor	26
7	Transporter	22
8	Lyase	13
9	Defense/immunity protein	10
10	Transfer/carrier protein	10
11	Calcium-binding protein	9
12	Enzyme modulator	7
13	Cell adhesion molecule	6
14	Cytoskeletal protein	4
15	Isomerase	4
16	Signaling molecule	3
17	Ligase	2
18	Chaperone	1
19	Extracellular matrix protein	1
20	Structural protein	1

as their similar ChEMBL compounds.

Information on the molecular targets of 75 compounds from SLT products was extracted from the ChEMBL database and targets of 66 compounds were retrieved. We found 402 unique human protein targets of 66 compounds which were further screened on the basis of their confidence scores. 364 unique human protein targets with confidence scores 8 and 9 were finally extracted for further analysis.

3.3. Functional annotation of the protein targets

The functional annotation of the 364 screened human protein targets of 66 compounds of SLT products was performed using PANTHER release 11.1. These protein targets were found to be associated with 97 biological pathways (Supplementary Table III). Out of these maximum number of protein targets belongs to the PANTHER protein class *receptors* which were followed by *transferases* and *hydrolases* (Table 1). The protein targets were found to be involved in 11 biological processes where processes such as *cellular process* (GO:0009987), *metabolic process* (GO:0008152), *response to stimulus* (GO:0050896) were associated with more than 100 targets (Fig. 2). The biological processes *reproduction* (GO:0000003) and *development process* (GO:0032502) also found to have potential to be affected from compounds of SLT products.

The association of the protein targets with diseases was studied using KEGG. A detailed list of diseases associated with potential SLT targets is provided in Supplementary Table IV. Briefly, 31 protein targets were associated with 32 types of congenital disorders of metabolism followed by 29 protein targets associated with 35 types of nervous system disorders (Fig. 3, Supplementary Table III; Table 1). 16 protein targets were also found to be associated with 51 types of cancers. The list include important and well characterized targets such as tumor protein 53 (TP53) and epidermal growth factor receptor (EGFR). We found that 13 compounds of SLT products such as coumarin, caffeine, flavonoid, cinnamon, methyl salicylate, 6,7-dihydroxycoumarin, benzo [a]pyrene, benzo[b]fluoranthene, benzo[e]pyrene, benzo[j]fluoranthene, benzo[k]fluoranthene, dieldrin and endrine target Epidermal Growth Factor receptor (EGFR). Of these, benzo[a]pyrene is group 1 carcinogen, dieldrin is group 2A carcinogen, benzo[b]fluoranthene, benzo[k]fluoranthene and benzo[j]fluoranthene are group 2B carcinogen, coumarin is group 3 carcinogen according to IARC monographs.

3.4. Toxicokinetic analysis

Toxicokinetic properties such as absorption, distribution, metabolism, excretion and toxicity (ADMET) were studied to identify the range of bioavailability, solubility, permeability, mutagenicity, and toxicity of different compounds present in SLT products.

As depicted in Fig. 4, 73 compounds of SLT products were predicted to have good absorption properties. Carcinogens such as NNN, NNK, NAT and NAB were predicted to be classified in good absorption category whereas other carcinogens such as benzo[a]pyrene predicted as moderate (Supplementary Table V). 77 compounds of SLT products were predicted to be showing high Caco-2 permeability. There were 67 compounds which were predicted to having optimum to good solubility properties.

On the basis of values of parameter Vdss, 23 compounds of SLT products were predicted to be highly distributed. Moreover, 62 compounds of SLT products are predicted to have very high to medium permeability to cross BBB as shown in Supplementary Table V. Fig. 4 shows that around 50% of compounds were predicted to have high CNS permeability.45 compounds including those classified as carcinogen/possible carcinogen by IARC are predicted to not bind to the plasma proteins. Also, 18 compounds were predicted to be inhibitors of CYP2D6.

Fifty percent of the compounds of SLT products were predicted to be mutagenic. Most of the carcinogenic compounds such as NNN, NNK, benzo[a]pyrene and all the compounds containing anthracenes and pyrenes were predicted as mutagen. More than 60 compounds of SLT products have been predicted as hepatotoxic as shown in Fig. 4.

These properties were also studied for their distribution in different disease types and it was found out that all compounds found to be associated with cancer, cardiovascular, congenital and immune diseases had good absorption properties while 41 out of 42 compounds associated with nervous system diseases had good absorption (Fig. 5). Of the 42 compounds associated with nervous system diseases, 23 were found to cross BBB and 21 were found to have the property of CNS permeability. A large proportion of compounds were also toxic and mutagenic.

3.5. Interaction network of compounds and targets

The interaction of compounds of SLT products and the protein targets has been illustrated in the network as shown in Fig. 6. Sub-networks were drawn for four prominent disease categories, namely nervous system disorders, cancer, cardiovascular diseases and immune system disorders. The node-degree distribution analysis shows higher degrees for nodes of cancer and nervous system disorders followed by cardiac and immune system diseases (Supplementary Figure I).

4. Discussion

With a substantial impact of SLT products on the health of the society, thorough understanding of the role of chemical compounds is absolute. We have provided the first comprehensive *in-silico* analysis of 180 chemical compounds present in SLT products and highlighted their associations with diseases. This study also identified associations with previously known diseases like cancer, nervous system diseases (stroke) and cardiovascular disorders which validate the current methodology. Simultaneously, it emphasizes the association of other categories of diseases which were not previously known to be associated with SLT products. These new categories include diseases of immune system, skin, digestive system, congenital disorders and degenerative disorders of the nervous system like Lewy body dementia (LBD), Huntington's disease-like syndrome and Schizophrenia. On analyzing the toxicokinetic properties of these chemicals, it was observed that most of them have good solubility and diffusion abilities along with mutagenic and toxic properties.

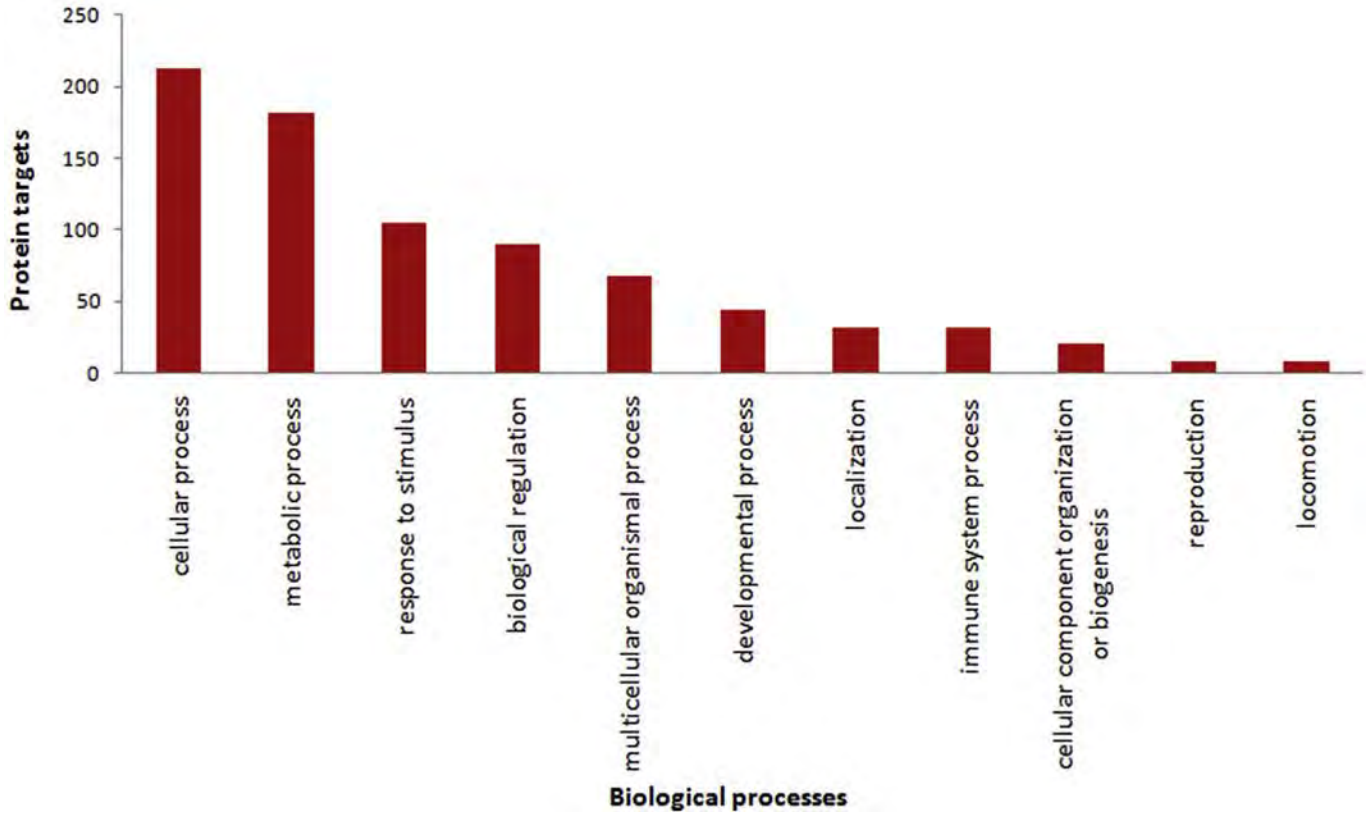

Fig. 2. Biological processes targeted by compounds of SLT products.

4.1. Absorption

Oral consumption of SLT products result in absorption of these chemicals leading to systemic circulation. Permeability and solubility are the two key parameters considered for absorption properties of a compound (Lagorce et al., 2017). As shown in Fig. 4, most of the compounds associated in different diseases have good absorption properties along with high Caco-2 permeability. This clearly indicates higher bioavailability of these chemicals upon tobacco consumption.

4.2. Distribution

Ability to cross the BBB and CNS permeability are the two factors considered to monitor the distribution of chemicals in the body. Efficacy of a distribution of any chemical compound is affected by the degree to which it binds to the proteins in blood. The more it is bound, the less efficiently it may traverse the cellular membranes or diffuse. Unbound compounds have the possibility of efficient movement and diffusion across cell membrane. Only 5% of one of the most important

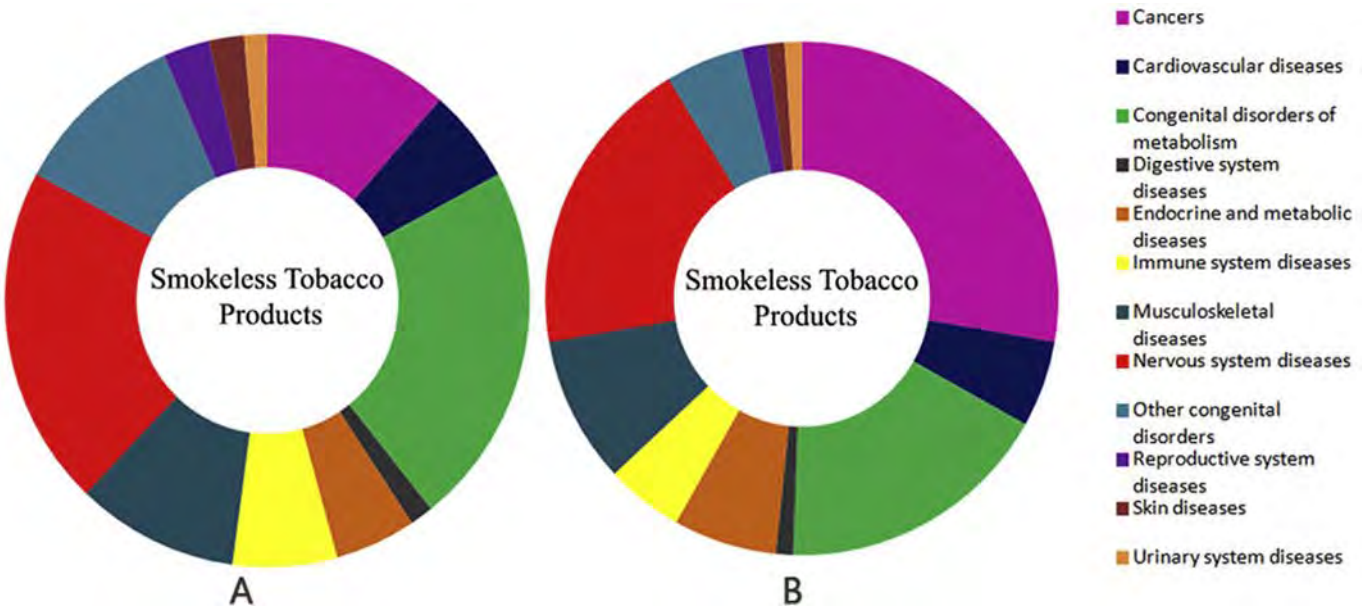

Fig. 3. A. Distribution of human protein targets of compounds of SLT products in various disease types. **B.** Distribution of subtypes of diseases associated with targets of compounds of SLT products.

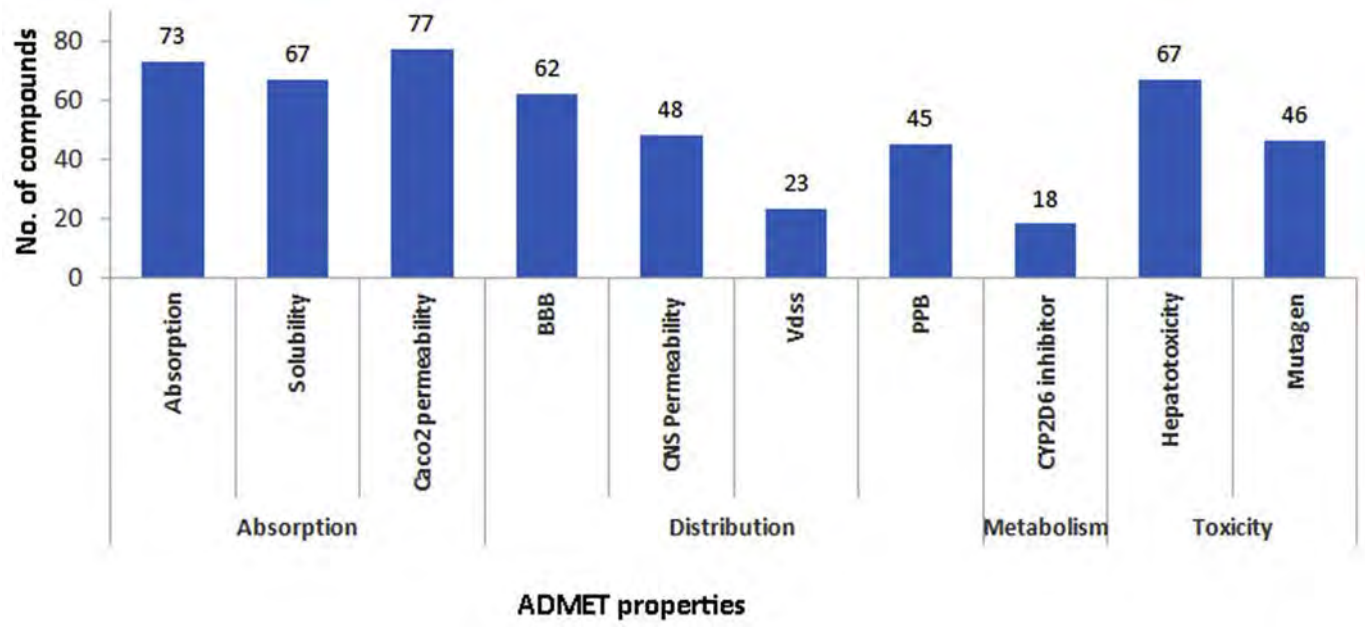

Fig. 4. Distribution of different ADMET properties in compounds present in SLT products (For reference values, please refer Supplementary Table II).

constituent of SLT products, nicotine, binds to plasma protein when it enters the human bloodstream which means that rest 95% is free for extensive distribution in the body (Benowitz et al., 2009). It was also observed from the ADMET analysis that around 50% of the compounds had the ability to penetrate the central nervous system along with strong possibility of crossing the blood-brain barrier (Fig. 3). Most of these chemicals were found to be associated with nervous system diseases, indicating a higher possibility of nervous side effects in SLT users.

4.3. Metabolism

Metabolism is the biotransformation of chemical compounds and xenobiotic compounds via metabolic pathway to their excretion. Cytochrome P450 mono-oxygenase (CYP) enzymes super family plays an important role in metabolism of large number of xenobiotics, especially isoforms such as 2D6, 2C9 and 3A4 (Lagorce et al., 2017). Many drugs are deactivated by this family of enzymes whose inhibition can

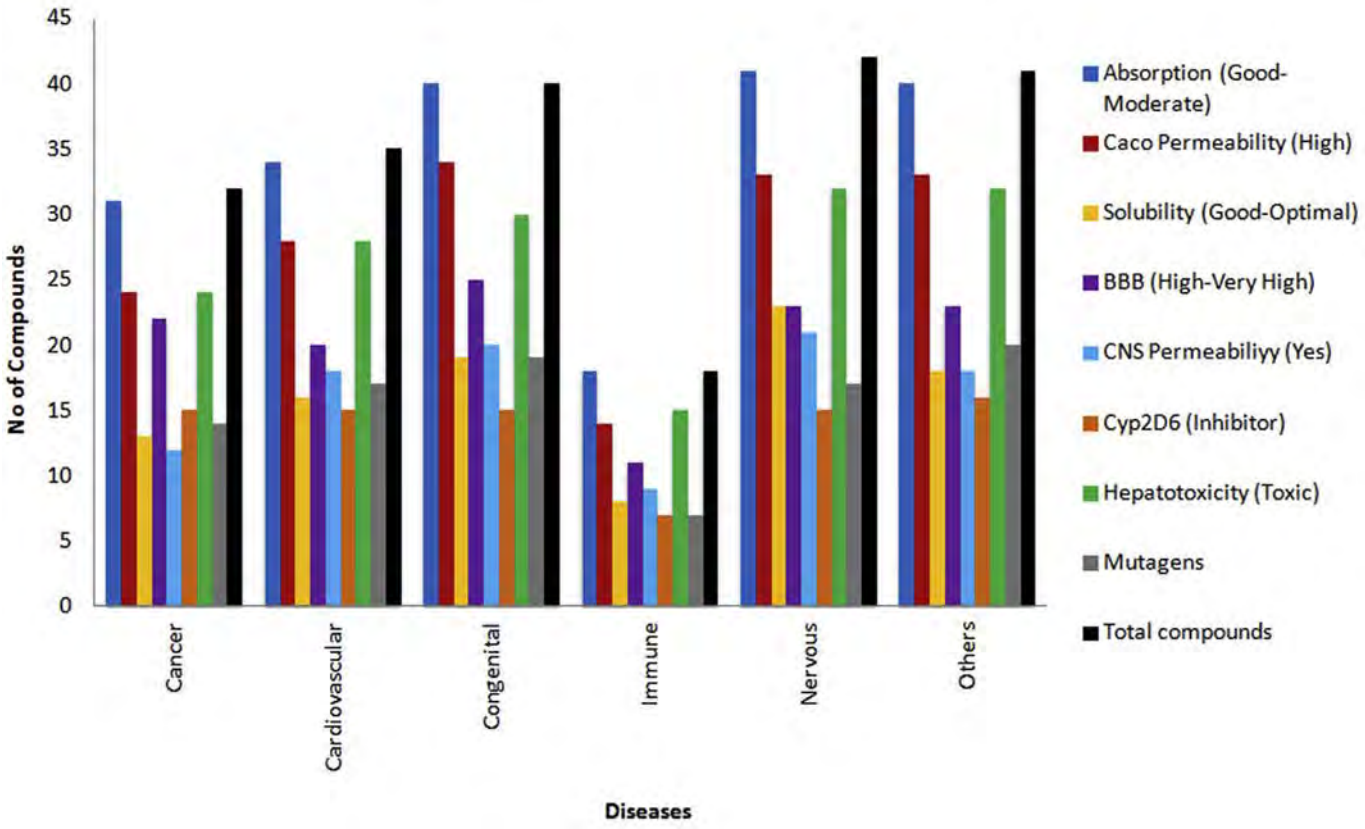

Fig. 5. Disease-wise distribution of different ADMET properties in present in SLT products (For reference values, please refer Supplementary Table II).

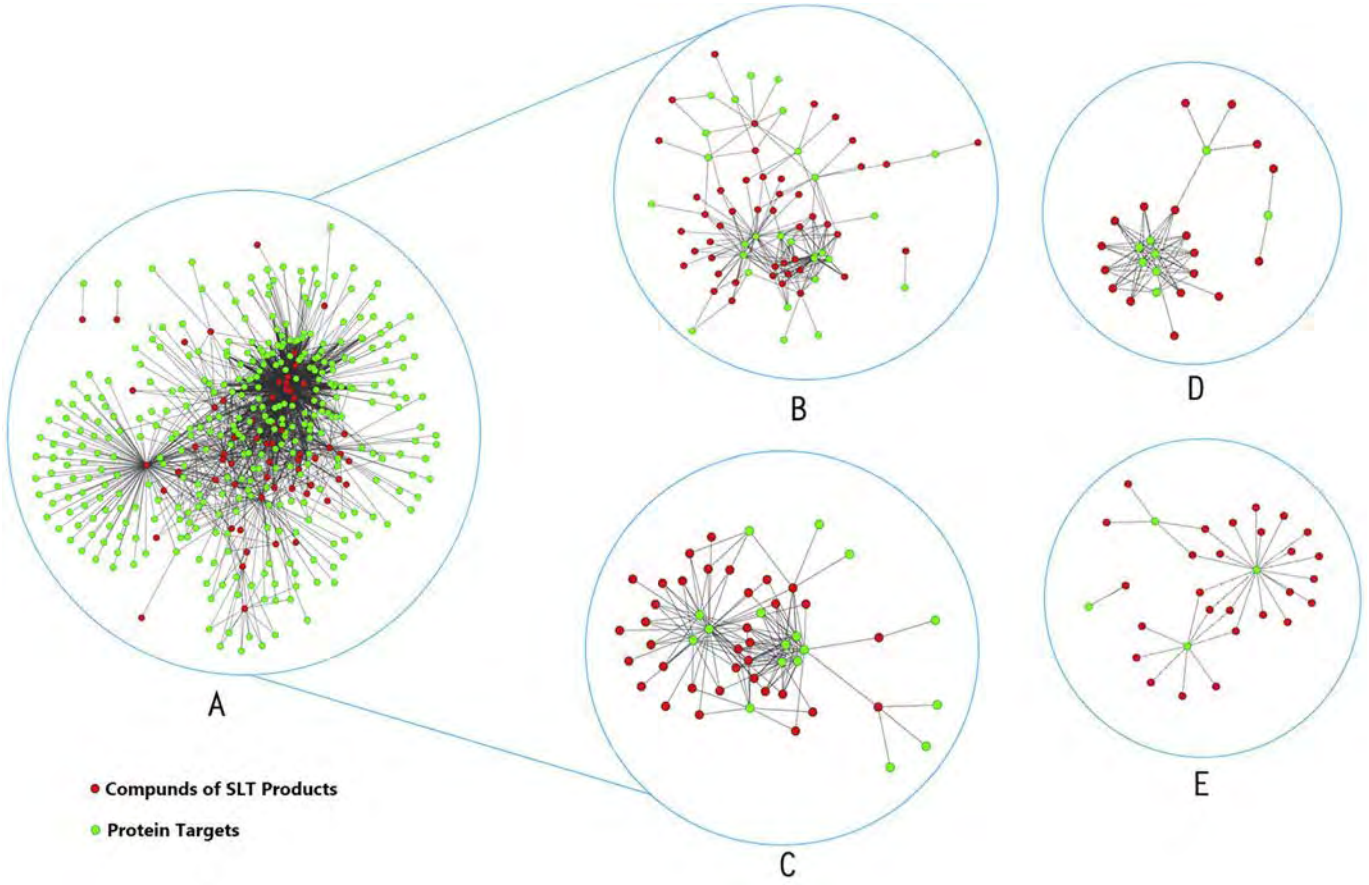

Fig. 6. (A) The interaction network of human protein targets and compound of SLT products. Sub-networks of compounds of SLT products with targets associated with **(B)** Nervous system disorders **(C)** Cancer **(D).** Cardiovascular diseases **(E)** Immune system diseases.

Table 2
ADMET properties of important compounds of SLT products (For reference values, please refer Supplementary Table II).

Compound	Class	Absorption	Solubility	Hepatotoxicity	Mutagenicity	Caco-2 permeability	CNS permeability
NNN	Tobacco specific nitrosamines	Good	Good	Toxic	Mutagen	High	Yes
NNK		Good	Good	Toxic	Mutagen	High	Yes
NAT		Good	Good	Toxic	Mutagen	High	Yes
NAB		Good	Good	Toxic	Mutagen	High	Yes
MNBA	N-Nitrosamino acids	Good	Optimal	Toxic	Mutagen	High	Yes
MNPA		Good	Optimal	Toxic	Mutagen	High	Yes
NAzCA		Good	Optimal	Toxic	Mutagen	High	Yes
NSAR		Good	Optimal	Toxic	Mutagen	Not predicted	Yes
NPIP	Volatile N-nitrosamines	Good	Good	Toxic	Mutagen	High	Yes
NMOR		Good	Optimal	Toxic	Mutagen	High	Yes
NDELA		Good	Too soluble	Toxic	Mutagen	High	Yes
NDMA		Good	Optimal	Toxic	Mutagen	Not predicted	Yes
5-Methyl chrysene	Poly aromatic hydrocarbons	Moderate	Very low but possible	Toxic	Mutagen	High	Yes
Benz[a]anthracene		Moderate	Very low but possible	Toxic	Mutagen	High	Yes
Benzo[a]pyrene		Moderate	Very low but possible	Toxic	Mutagen	High	Yes
Benzo[b]fluoranthene		Moderate	Very low but possible	Toxic	Mutagen	High	Yes
Benzo[k]fluoranthene		Moderate	Very low but possible	Toxic	Mutagen	High	Yes
Benzo[j]fluoranthenes		Moderate	Very low but possible	Toxic	Mutagen	High	Yes
Benzo[e]pyrene		Moderate	Very low but possible	Toxic	Mutagen	Not predicted	Yes

lead to undesirable drug metabolism. Therefore, to identify the side effects of any chemical compound, it is important to measure its ability to inhibit cytochrome P450. 18 compounds were found to be CYP2D6 inhibitors.

Around 40% of compounds associated in different diseases such as cancer, cardiovascular, congenital and immune disorders were predicted as CYP2D6 inhibitors. This indicates the undesirable affect of these compounds in metabolism of SLT users.

4.4. Toxicity

Toxicity is a phenomenon in which a substance has the ability to damage cells and organs. Toxicity of compounds of SLT products has been monitored by considering their hepatotoxic effect and mutagenic properties. A large number of chemical compounds of SLT products were found to be hepatotoxic. These compounds were also predicted to be involved in various diseases where around 75% of compounds associated with cancer, cardiovascular, congenital, immune and nervous

system disorders were hepatotoxic.

NNN and NNK are known to cause DNA adducts and mutations which lead to tumor growth and induce carcinogenesis (Peterson, 2010). The formation of DNA adducts is crucial and further involves tumor suppressor genes, oncogenes and other processes (Huang et al., 2011; Xue et al., 2014). We observed that around 45% of total compounds were predicted as mutagens. Many of these compounds were associated with different cancers. However, many compounds were also found to be involved in other diseases such as cardiovascular, congenital and nervous system disorders. This further indicates the complications which may get aggravated with SLT use.

Considering the toxicokinetic properties, 19 toxic and mutagenic compounds of SLT products were identified which had good absorption, solubility, permeability and were predicted to cross the central nervous system (Table 2). These compounds include the most common TSNAs, NNN, NNK, N′-nitrosoanatabine (NAT) and N′-nitrosoanabasine (NAB) as well as various compound of other classes such N-Nitrosamino acids, Volatile N-nitrosamines and Poly aromatic hydrocarbons.

On studying the proteins targeted by these compounds, it was observed that targets of most of these compounds are found to belong to *receptor proteins. Among* receptors, prominently affected are the *D3 and D4 Dopamine receptors* which are known neurotransmitters and participate in neurodegenerative disorders such as Huntington's disease (Rangel-Barajas et al., 2015). The second most common class of protein target identified is *transferases* whose members (proteins from kinase family) are known to be highly implicated in neurodegenerative disorders (Svenson et al., 2004). The cyclin dependent kinases which include serine/threonine kinases are known to regulate cellular processes such as cell cycle and neuronal differentiation and are associated with pathogenesis of neurodegenerative diseases (Monaco and Vallano, 2005). Thus, the role of these chemicals in neurodegenerative diseases needs to be investigated in detail.

EGFR mediates cell proliferation, survival and differentiation and its misregulation leads to a variety of cancers (Sasaki et al., 2013). Activation of EGFR requires certain ligands and its hyper activation is associated with cancers of breast, ovary, head and neck, colorectal, prostate, cervix etc (Ceresa and Vanlandingham, 2008). Compounds present in SLT products have been found to target EGFR suggesting their probable role of in regulation of EGFR and its cancerous properties. Thus, even though the carcinogenic properties of SLT products have been studied, their intricacies still need to be explored.

Tumor protein p53 is among the well studied proteins involved in cancer progression where its role in tumor suppression is compromised by damaging the genes or mutating by chemicals or radiations. The poly-aromatic hydrocarbon benzo[a]pyrene is also known to target p53 gene and inactivates its function as tumor suppression. Many of the compounds of SLT products are shown in the present study to target protein p53 suggesting their role in cancer progression (Chen et al., 1998; Pfeifer et al., 2002). SLT products are also known to be involved in reproductive outcomes such as low birth weight and still birth but the underlying mechanisms are not clear.

One of the major limitations of this study was non-availablility of complete information about the chemical composition of SLT products. Also, this study could not take into account the nature of relation (agonist or antagonist) between the chemicals and proteins involved in diseases. Other limitations include limited amount of quality data in ADMET model designing. In conclusion, this study gives insights to harmful effects associated with the chemicals present in SLT products which needs to be supported with experimental evidence.

5. Conclusion

This study predicted many diseases not previously known to be associated with consumption of SLT products. It was further supported by toxicokinetic analysis of these chemicals and needs validation by experimental analysis. This study provides the first evidence for establishing priorities in research on chemical profiling of SLT products. In addition, it also provides initial data for developing regulations, guidelines and policies on chemical composition of SLT products. Some of the future directions include incorporating other classes of chemical compounds and developing a quantitative relation between chemicals and their associated health effects. We hope that the present study will initiate research in this important aspect of public health and open new avenues for researchers. We hope that this provides the policy makers a much needed insights into the gaps in the chemical profiling of smokeless tobacco products and aid in the development of regulatory policies.

Conflicts of interest

None.

Ethics approval

Not Applicable.

Funding

This work was supported by two projects: Second phase of task force Biomedical Informatics Centers of ICMR (Project No. BIC/12(06)/2013) and WHO FCTC Global Knowledge Hub on Smokeless Tobacco (Reference No 2016/643768-0).

Acknowledgement

Special thanks to Prof. Punit Kaur, Department of Biophysics, All India Institute of Medical Sciences, New Delhi for software support in calculating the ADMET properties.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.yrtph.2018.03.002.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.yrtph.2018.03.002.

References

Adams, J.D., LaVoie, E.J., Hoffmann, D., 1985. On the pharmacokinetics of tobacco-specific N-nitrosamines in Fischer rats. Carcinogenesis 6, 509–511. https://doi.org/3986958.

Adams, J.D., Lavoie, E.J., O'Donnell, M., Hoffmann, D., 1984. Pharmacokinetics of tobacco-specific N-nitrosamines. IARC Sci. Publ. 779–785.

Benowitz, N.L., Hukkanen, J., Jacob, P., 2009. Nicotine chemistry, metabolism, kinetics and biomarkers. Nicotine Psychopharmacol 192, 29–60. https://doi.org/10.1007/978-3-540-69248-5_2.

Boffetta, P., Straif, K., 2009. Use of smokeless tobacco and risk of myocardial infarction and stroke: systematic review with meta-analysis. BMJ 339, b3060.

Borgerding, M.F., Bodnar, J.A., Curtin, G.M., Swauger, J.E., 2012. The chemical composition of smokeless tobacco: a survey of products sold in the United States in 2006 and 2007. Regul. Toxicol. Pharmacol. 64, 367–387. https://doi.org/10.1016/j.yrtph.2012.09.003.

Brunnemann, K.D., Genoble, L., Hoffmann, D., 1985. N-Nitrosamines in chewing tobacco: an international comparison. J. Agric. Food Chem. 33, 1178–1181. https://doi.org/10.1021/jf00066a039.

Ceresa, B.P., Vanlandingham, P.A., 2008. Molecular mechanisms that regulate epidermal growth factor receptor inactivation. Clin. Med. Oncol. 2, 47–61.

Chen, J.X., Zheng, Y., West, M., Tang, M.S., 1998. Carcinogens preferentially bind at methylated CpG in the p53 mutational hot spots. Canc. Res. 58, 2070–2075.

Gaulton, A., Bellis, L.J., Bento, A.P., Chambers, J., Davies, M., Hersey, A., Light, Y., McGlinchey, S., Michalovich, D., Al-Lazikani, B., Overington, J.P., 2012. ChEMBL: a large-scale bioactivity database for drug discovery. Nucleic Acids Res. 40, D1100–D1107. https://doi.org/10.1093/nar/gkr777.

Gupta, B., Johnson, N.W., 2014. Systematic review and meta-analysis of association of smokeless tobacco and of betel quid without tobacco with incidence of oral cancer in

South Asia and the Pacific. PLoS One 9 e113385. https://doi.org/10.1371/journal.pone.0113385.
Huang, R.-Y., Li, M.-Y., Hsin, M.K.Y., Underwood, M.J., Ma, L.T., Mok, T.S.K., Warner, T.D., Chen, G.G., 2011. 4-Methylnitrosamino-1-3-pyridyl-1-butanone (NNK) promotes lung cancer cell survival by stimulating thromboxane A2 and its receptor. Oncogene 30, 106–116. https://doi.org/10.1038/onc.2010.390.
IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 2012. Personal Habits and Indoor Combustions. [WWW Document]. 100E. URL http://monographs.iarc.fr/ENG/Monographs/vol100E/mono100E.pdf.
IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 2007. Smokeless Tobacco and Some Tobacco-specific N-nitrosamines. [WWW Document]. vol.89. URL http://monographs.iarc.fr/ENG/Monographs/vol85/mono85.pdf.
IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 2004. Betel-quid and Areca-nut Chewing and Some Areca-nut-derived Nitrosamines. [WWW Document]. vol.85. URL http://monographs.iarc.fr/ENG/Monographs/vol85/mono85.pdf.
Idris, A.M., Nair, J., Ohshima, H., Friesen, M., Brouet, I., Faustman, E.M., 1991. Unusually high levels of carcinogenic tobacco-specific nitrosamines in Sudan snuff(toombak). Carcinogenesis 12, 1115–1118. https://doi.org/10.1.1.1000.6382.
Jaccard, P., 1901. Distribution de la flore alpine dans le Bassin des Dranses et dans quelques régions voisines. Bull. del la Société Vaudoise des Sci. Nat 37, 241–272.
Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., Morishima, K., 2017. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res. 45, D353–D361. https://doi.org/10.1093/nar/gkw1092.
Kanehisa, M., Goto, S., 2000. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 28, 27–30.
Klus, H., Kunze, M., Koenig, S., Poeschl, E., 2014. Smokeless tobacco - an overview. Beiträge zur Tab./Contrib. Tob. Res. 23. https://doi.org/10.2478/cttr-2013-0865.
Krause, S., Willighagen, E., Steinbeck, C., 2000. JChemPaint - using the collaborative forces of the internet to develop a free editor for 2D chemical structures. Molecules 5, 93–98. https://doi.org/10.3390/50100093.
Lagorce, D., Douguet, D., Miteva, M.A., Villoutreix, B.O., 2017. Computational analysis of calculated physicochemical and ADMET properties of protein-protein interaction inhibitors. Sci. Rep. 7 46277. https://doi.org/10.1038/srep46277.
Monaco, E.A., Vallano, M. Lou, 2005. Role of protein kinases in neurodegenerative disease: cyclin-dependent kinases in Alzheimer's disease. Front. Biosci 10, 143–159.
National Cancer Institute and Centers for Disease Control and Prevention, Bethesda, M., 2014. Smokeless tobacco and public health: a global perspective, NIH public. In: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention and National Institutes of Health, National Cancer Institute, Bethesda, MD.
O'Boyle, N.M., Banck, M., James, C.A., Morley, C., Vandermeersch, T., Hutchison, G.R., 2011. Open Babel: an open chemical toolbox. J. Cheminform 3, 33. https://doi.org/10.1186/1758-2946-3-33.
Peterson, L.A., 2010. Formation, repair, and genotoxic properties of bulky DNA adducts formed from tobacco-specific nitrosamines. J. Nucleic Acids 2010, 1–11. https://doi.org/10.4061/2010/284935.
Pfeifer, G.P., Denissenko, M.F., Olivier, M., Tretyakova, N., Hecht, S.S., Hainaut, P., 2002. Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. Oncogene 21, 7435–7451. https://doi.org/10.1038/sj.onc.1205803.
Piano, M.R., Benowitz, N.L., Fitzgerald, G.A., Corbridge, S., Heath, J., Hahn, E., Pechacek, T.F., Howard, G., American heart association council on cardiovascular nursing, 2010. Impact of smokeless tobacco products on cardiovascular disease: implications for policy, prevention, and treatment: a policy statement from the American Heart Association. Circulation 122, 1520–1544. https://doi.org/10.1161/CIR.0b013e3181f432c3.
Pires, D.E.V., Blundell, T.L., Ascher, D.B., 2015. pkCSM: predicting small-molecule pharmacokinetic and toxicity properties using graph-based signatures. J. Med. Chem. 58, 4066–4072. https://doi.org/10.1021/acs.jmedchem.5b00104.
Ponnan, P., Gupta, S., Chopra, M., Tandon, R., Baghel, A.S., Gupta, G., Prasad, A.K., Rastogi, R.C., Bose, M., Raj, H.G., 2013. 2D-QSAR, docking studies, and in silico ADMET prediction of polyphenolic acetates as substrates for protein acetyltransferase function of glutamine synthetase of Mycobacterium tuberculosis. ISRN Struct. Biol. 2013, 1–12. https://doi.org/10.1155/2013/373516.
Pool-Zobel, B.L., Liegibel, U., Klein, R.G., Schmezer, P., 1991. Toxicokinetics of nicotine-derived nitrosamines in the rat. In: Effects of Nicotine on Biological Systems. Birkhäuser Basel, Basel, pp. 115–121. https://doi.org/10.1007/978-3-0348-7457-1_16.
Rangel-Barajas, C., Coronel, I., Florán, B., 2015. Dopamine receptors and neurodegeneration. Aging Dis 6, 349–368. https://doi.org/10.14336/AD.2015.0330.
Rickert, W.S., Joza, P.J., Trivedi, A.H., Momin, R.A., Wagstaff, W.G., Lauterbach, J.H., 2009. Chemical and toxicological characterization of commercial smokeless tobacco products available on the Canadian market. Regul. Toxicol. Pharmacol. 53, 121–133. https://doi.org/10.1016/j.yrtph.2008.12.004.
Sasaki, T., Hiroki, K., Yamashita, Y., 2013. The role of epidermal growth factor receptor in cancer metastasis and microenvironment. BioMed Res. Int. 2013, 1–8. https://doi.org/10.1155/2013/546318.
Shah, G., Chaturvedi, P., Vaishampayan, S., 2012. Arecanut as an emerging etiology of oral cancers in India. Indian J. Med. Paediatr. Oncol. 33, 71–79. https://doi.org/10.4103/0971-5851.99726.
Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504. https://doi.org/10.1101/gr.1239303.
Sharan, R.N., Mehrotra, R., Choudhury, Y., Asotra, K., 2012. Association of betel nut with carcinogenesis: revisit with a clinical perspective. PLoS One 7, e42759. https://doi.org/10.1371/journal.pone.0042759.
Siddiqi, K., Shah, S., Abbas, S.M., Vidyasagaran, A., Jawad, M., Dogar, O., Sheikh, A., 2015. Global burden of disease due to smokeless tobacco consumption in adults: analysis of data from 113 countries. BMC Med. 13, 194. https://doi.org/10.1186/s12916-015-0424-2.
Siminszky, B., Gavilano, L., Bowen, S.W., Dewey, R.E., 2005. Conversion of nicotine to nornicotine in Nicotiana tabacum is mediated by CYP82E4, a cytochrome P450 monooxygenase. Proc. Natl. Acad. Sci. U. S. A 102, 14919–14924. https://doi.org/10.1073/pnas.0506581102.
Sinha, D.N., Abdulkader, R.S., Gupta, P.C., 2016. Smokeless tobacco-associated cancers: a systematic review and meta-analysis of Indian studies. Int. J. cancer 138, 1368–1379. https://doi.org/10.1002/ijc.29884.
Sinha, D.N., Suliankatchi, R.A., Gupta, P.C., Thamarangsi, T., Agarwal, N., Parascandola, M., Mehrotra, R., 2018. Global burden of all-cause and cause-specific mortality due to smokeless tobacco use: systematic review and meta-analysis. Tob. Control 27, 35–42. https://doi.org/10.1136/tobaccocontrol-2016-053302.
Snyder, R.D., Pearl, G.S., Mandakas, G., Choy, W.N., Goodsaid, F., Rosenblum, I.Y., 2004. Assessment of the sensitivity of the computational programs DEREK, TOPKAT, and MCASE in the prediction of the genotoxicity of pharmaceutical molecules. Environ. Mol. Mutagen. 43, 143–158. https://doi.org/10.1002/em.20013.
Stanfill, S.B., Oliveira da Silva, A.L., Lisko, J.G., Lawler, T.S., Kuklenyik, P., Tyx, R.E., Peuchen, E.H., Richter, P., Watson, C.H., 2015. Comprehensive chemical characterization of Rapé tobacco products: nicotine, un-ionized nicotine, tobacco-specific N'-nitrosamines, polycyclic aromatic hydrocarbons, and flavor constituents. Food Chem. Toxicol. 82, 50–58. https://doi.org/10.1016/j.fct.2015.04.016.
Stepanov, I., Jensen, J., Hatsukami, D., Hecht, S.S., 2008. New and traditional smokeless tobacco: comparison of toxicant and carcinogen levels. Nicotine Tob. Res. 10, 1773–1782. https://doi.org/10.1080/14622200802443544.
Stepanov, I., Villalta, P.W., Knezevich, A., Jensen, J., Hatsukami, D., Hecht, S.S., 2010. Analysis of 23 polycyclic aromatic hydrocarbons in smokeless tobacco by gas Chromatography – Mass spectrometry. Chem. Res. Toxicol. 23, 66–73. https://doi.org/10.1021/tx900281u.
Svenson, I.K., Kloos, M.T., Gaskell, P.C., Nance, M.A., Garbern, J.Y., Hisanaga, S., Pericak-Vance, M.A., Ashley-Koch, A.E., Marchuk, D.A., 2004. Intragenic modifiers of hereditary spastic paraplegia due to spastin gene mutations. Neurogenetics 5, 157–164. https://doi.org/10.1007/s10048-004-0186-z.
Tanimoto, T.T., 1957. IBM Internal Report 17th Nov.
Thomas, P.D., Campbell, M.J., Kejariwal, A., Mi, H., Karlak, B., Daverman, R., Diemer, K., Muruganujan, A., Narechania, A., 2003. PANTHER: a library of protein families and subfamilies indexed by function. Genome Res. 13, 2129–2141. https://doi.org/10.1101/gr.772403.
Thun, M.J., Jemal, A., 2003. IARC classification of carcinogens. In: Kufe, D.W., Pollock, R.E., Weichselbaum, R.R. (Eds.), Holland-Frei Cancer Medicine, sixth ed. BC Decker, Hamilton (ON). https://www.ncbi.nlm.nih.gov/books/NBK12823/.
Tricker, A.R., Preussmann, R., 1988. The occurrence of N-nitro compounds in zarda tobacco. Canc. Lett. 42, 113–118. https://doi.org/10.1016/0304-3835(88)90247-9.
U.S. Department of Health and Human Services, National Institutes of Health, N.C.I., 2014. Smokeless tobacco or health : an international perspective. [Rockville, Md.] In: U.S. Dept. of Health and Human Services, Public Health Service, National Institutes of Health, [National Cancer Institute], [1992].
Verma, S., Yadav, S., Singh, I., 2010. Trace metal concentration in different Indian tobacco products and related health implications. Food Chem. Toxicol. 48, 2291–2297. https://doi.org/10.1016/j.fct.2010.05.062.
Xue, J., Yang, S., Seng, S., 2014. Mechanisms of cancer induction by tobacco-specific NNK and NNN. Cancers 6, 1138–1156. https://doi.org/10.3390/cancers6021138.

PLOS ONE

RESEARCH ARTICLE

Vapors Produced by Electronic Cigarettes and E-Juices with Flavorings Induce Toxicity, Oxidative Stress, and Inflammatory Response in Lung Epithelial Cells and in Mouse Lung

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OPEN ACCESS

Citation: Lerner CA, Sundar IK, Yao H, Gerloff J, Ossip DJ, McIntosh S, et al. (2015) Vapors Produced by Electronic Cigarettes and E-Juices with Flavorings Induce Toxicity, Oxidative Stress, and Inflammatory Response in Lung Epithelial Cells and in Mouse Lung. PLoS ONE 10(2): e0116732. doi:10.1371/journal.pone.0116732

Academic Editor: M. Firoze Khan, University of Texas Medical Branch, UNITED STATES

Received: September 12, 2014

Accepted: December 12, 2014

Published: February 6, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by the National Institute of Drug Abuse at the National Institutes of Health, NIH (R21DA036057 to RR and IR), NIH 2R01 HL085613 (IR), 1R01HL092842 (IR), and by the National Cancer Institute, NCI at the NIH (R01CA132950 to DJO, and R01CA152093 to SM), pulmonary training grant T32 HL066988, and by the University of Rochester Clinical Translational Research Institute, CTSI 5UL1RR024160 incubator

Abstract

Oxidative stress and inflammatory response are the key events in the pathogenesis of chronic airway diseases. The consumption of electronic cigarettes (e-cigs) with a variety of e-liquids/e-juices is alarmingly increasing without the unrealized potential harmful health effects. We hypothesized that electronic nicotine delivery systems (ENDS)/e-cigs pose health concerns due to oxidative toxicity and inflammatory response in lung cells exposed to their aerosols. The aerosols produced by vaporizing ENDS e-liquids exhibit oxidant reactivity suggesting oxidants or reactive oxygen species (OX/ROS) may be inhaled directly into the lung during a "vaping" session. These OX/ROS are generated through activation of the heating element which is affected by heating element status (new versus used), and occurs during the process of e-liquid vaporization. Unvaporized e-liquids were oxidative in a manner dependent on flavor additives, while flavors containing sweet or fruit flavors were stronger oxidizers than tobacco flavors. In light of OX/ROS generated in ENDS e-liquids and aerosols, the effects of ENDS aerosols on tissues and cells of the lung were measured. Exposure of human airway epithelial cells (H292) in an air-liquid interface to ENDS aerosols from a popular device resulted in increased secretion of inflammatory cytokines, such as IL-6 and IL-8. Furthermore, human lung fibroblasts exhibited stress and morphological change in response to treatment with ENDS/e-liquids. These cells also secrete increased IL-8 in response to a cinnamon flavored e-liquid and are susceptible to loss of cell viability by ENDS e-liquids. Finally, exposure of wild type C57BL/6J mice to aerosols produced from a popular e-cig increase pro-inflammatory cytokines and diminished lung glutathione levels which are critical in maintaining cellular redox balance. Thus, exposure to e-cig aerosols/juices incurs measurable oxidative and inflammatory responses in lung cells and tissues that could lead to unrealized health consequences.

project (IR). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

The consumption of electronic nicotine delivery systems (ENDS) and electronic cigarettes (e-cigs) is rising and currently scientific information necessary to inform the FDA and clinicians of potential health risks is lacking. Studies involving the effects of ENDS/e-cig liquids and aerosols on animal cells and tissues, in particular those of the lung, are lacking and the long-term outcome of chronic ENDS use is difficult to predict. Oxidative toxicity and inflammation are associated with increased risk of lung diseases caused by conventional tobacco products is well established [1]. However, there is no clear indication that inhaling aerosols from ENDS/e-cigs (as a cessation device) will allow a healthy outcome for users and furthermore, the manufactures that produce ENDS globally are not liable to disclose the materials and chemicals employed in their fabrication.

Two independent studies have reported that certain flavored e-liquids exhibit differential *in vitro* cytotoxicity when applied directly to various cells independent of nicotine, suggesting potential toxicities are associated with flavor additives [2,3]. Other toxic chemicals including carcinogens which are not typically found in e-liquids may be released or generated from ENDS/e-cigs and have been detected at low levels in various ENDS aerosols [4–6]. Some of these toxicants may emanate from heated structural materials while drawing air through an ENDS device, but are also proposed to form during the vaporization process [7,8]. Specific particulates, heavy metals, and toxic carbonyls in ENDS/e-cig aerosols have recently been measured in e-cigs aerosols as well [5,7,9,10].

Despite limited evidence that ENDS/e-cigs pose a danger, there is debate as to whether meaningful comparisons exist between the health risks of those exposed to tobacco smoke and those exposed to aerosols generated by ENDS devices [11]. Many of the secondary compounds (polyaromatic hydrocarbons, PAHs, aldehydes, and carbonyls) identified in ENDS aerosols and replacement liquids (e-liquids) are considered low level, especially in comparison to levels measured in environmental tobacco/cigarette smoke [5–7,12,13]. Furthermore, the levels of toxic compounds identified in ENDS aerosols that primary users would be exposed to in a "vaping" session are also not expected to approach established threshold limit values for what is considered a health risk for by-standard exposure to these compounds in cigarette smoke (passive smoking/second hand smoke) [14]. However, oxidants/reactive oxygen species (OX/ROS) found in cigarette smoke and generated from tars are major contributors in mediating an inflammatory state, which have been implicated in the pathogenesis of diseases, such as chronic obstructive pulmonary disease (COPD) and lung cancer [15]. The presence or generation of OX/ROS associated with ENDS devices and e-liquids has yet to be evaluated and may pose a health risk that is underappreciated.

There are approximately 10^{15} free radicals in a puff of conventional cigarette smoke in addition to heavy metals nanoparticles which have also recently been shown in e-cig aerosols to similar levels per "puff" [10,16]. Heavy metals may undergo redox cycling and alter the oxidation state of the cell by potentiating the production of ROS [17]. It is expected that OX/ROS in aerosols of ENDS/e-cigs will have an impact on cellular oxidative stress, redox imbalance, and lung inflammation, but this is still not clear *in vitro* in lung cells and *in vivo* in lungs. We hypothesized that electronic nicotine delivery systems (ENDS)/e-cigs induce oxidative toxicity and inflammatory response by generation of ROS and alteration in redox GSH levels in lung cells *in vitro* and *in vivo* in mouse lung exposed to their aerosols, respectively.

We determined the source of oxidants produced from ENDS/e-cigs by a modified 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescein derived dye to detect OX/ROS reactivity in ENDS/e-cig aerosols and pre-vaporized e-liquids in a cell free system. We also evaluated cultured lung cells exposed to e-liquids or aerosols for cell toxicity, inflammation,

and then extended our studies to a mouse model of e-cig aerosol exposure. By exposing wild-type (C57BL/6J) mice to e-cig aerosols, we examined the effect of short-term (3 days) exposure to e-cig aerosols on aspects of lung inflammation, oxidative stress, and redox physiology by measuring changes in glutathione levels.

Materials and Methods

Ethics statement

All experimental protocols were performed in accordance with the standards established by the United States Animal Welfare Act, as set forth by the National Institutes of Health guidelines. The research protocol for these studies was approved by the University of Rochester Committee on Animal Research.

Materials

Two ENDS devices were used. First, a refillable pen style ENDS (eGo Vision Spinner battery, China) and compatible clearomizer chamber (Anyvape, China) with 2.2 ohm heating element was purchased from local retailers (**Fig. 1A**). The clearomizer chamber can be easily filled with e-liquid of choice allowing liquid to continuously absorb into heating element wick. Refillable e-liquids (**Table 1**) for use with refillable ENDS were purchased from various local retailers. Second, is the Blu e-cigs where the cartomizer is manufactured to be disposable when the pre-loaded e-liquid is exhausted. The Blu e-cigs and disposable cartomizer cartridges were purchased from local retailers (**Fig. 1A**).

Cell-free ROS assay

The relative levels of OX/ROS produced from e-cig vapor or smoke (Federal Trade Commission protocol) using a CSM-SSM machine (CH-Technologies Inc.) from filtered research grade cigarettes (3R4F) was determined using 2',7'dichlorofluorescein diacetate (H2 DCF-DA) fluorogenic probe (EMD Bioscience, CA) as described previously [18,19]. In brief, aerosols generated from ENDS/e-cigs were tested for OX/ROS passing through tubing over a distance similar to that from the mouth to the bifurcation of the human trachea (approximately 20–24 cm) [20]. For each exposure, 5 ml of dichlorofluorescein DCFH-HRP solution [21,22], was loaded into a clean glass bubbler (Prism research). A lab pump (FMI, Syosset, NY) with a flow range of 0-1296 ml/min was switch activated using an FMI stroke rate controller set at 60% flow to draw a steady stream of e-cig aerosols/cigarette smoke directly through the DCFH solution. E-cig aerosols were pulsed through DCFH in the bubbler at room temperature for 4–5 seconds [23] at 30 second intervals for a total of 10 minutes. Following exposures, sample tubes were placed on ice and protected from light sources until analysis. A spectrofluorometer (Turner Quantech fluorometer Model FM109535 from Barnstead International/Thermolyne Corporation) was used to measure oxidized dichlorofluorescein (DCF) fluorescence at absorbance/emission maxima of 485 nm/535 nm. Hydrogen peroxide standards between 0 and 50 μM were created from 1 M stock and reacted at room temperature for 10 minutes with prepared DCFH solution in a total of 5 ml. These standards were then used to calibrate fluorescence intensity units (FIU) which numerically match respective hydrogen peroxide (H_2O_2) concentrations. The DCF fluorescence data are expressed as μM H_2O_2 equivalents referring to the concentration of the H_2O_2 added to the DCFH solution [16,19,24].

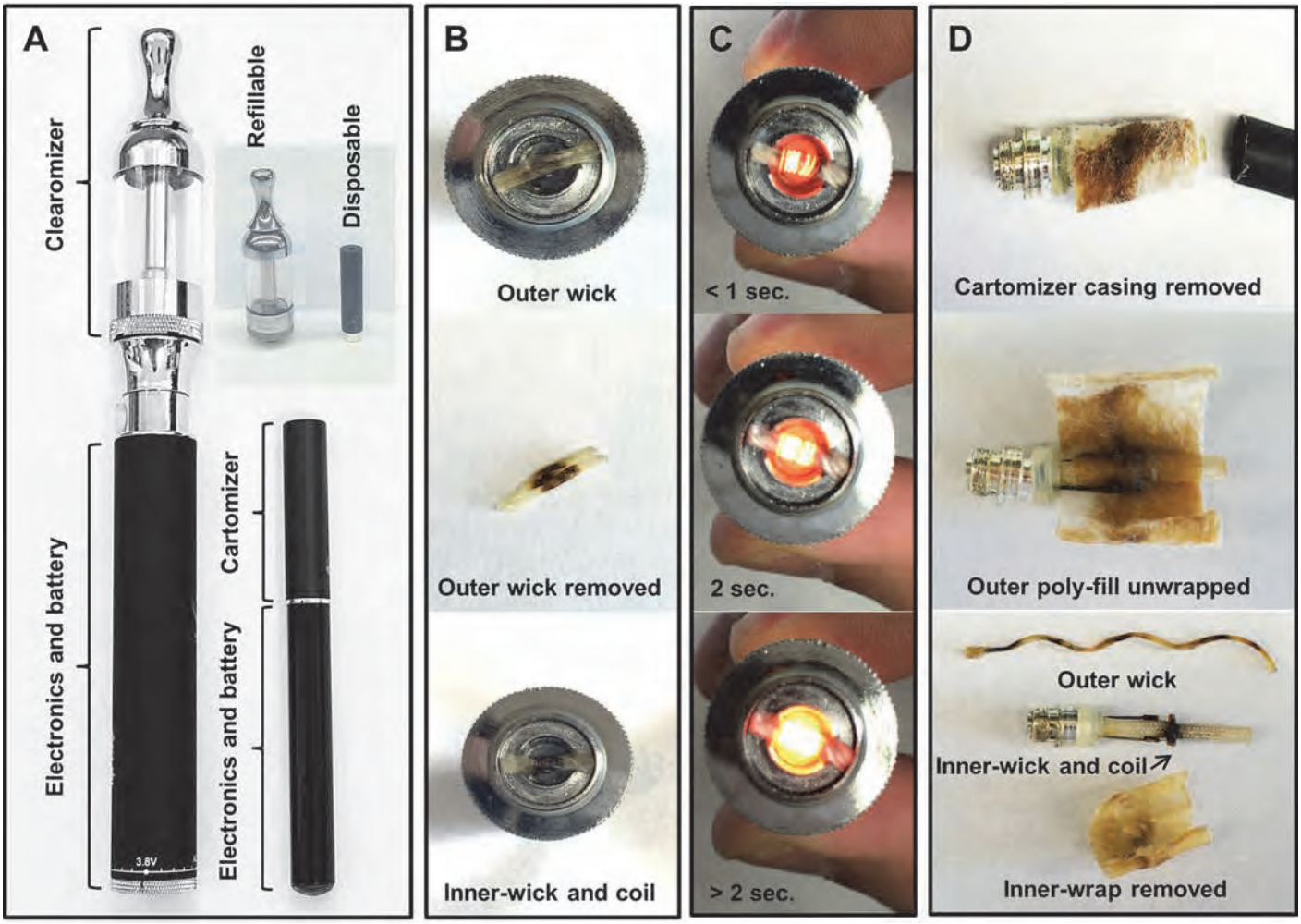

Fig 1. Refillable ENDS and Blu e-cigarettes. (A) Refillable ENDS and Blu e-cigarettes (B) Clearomizer removed, outer wick shown covering top of heating element coil (Upper panel). Used wick removed showing darkened region that contacted heating coil (Middle panel). Heating coil wrapped around second wick (Lower panel). (C) Activating heating element on refillable ENDS with Clearomizer removed. Less than 1 second activation (Upper panel), 2 seconds activation (Middle panel), greater than 2 second's activation (Lower panel). (D) Cartomizer casing removed after previous use (Upper panel). Poly-fill material with partially absorbed e-liquid wrapped around the core. Outer material removed exposing inner absorbent material tightly wrapped around heating element (Middle panel). Heating element exposed showing coil wrapped wick secured perpendicular to longer woven polymer tubing. A long thin fiber that was wrapped around the heating coil shows points of contact with coil wire (Lower panel).

doi:10.1371/journal.pone.0116732.g001

Cell culture and treatments

Human bronchial airway epithelial cells (H292) and human fetal lung fibroblasts (HFL1) were obtained from American Type Culture Collection (Manassas, VA). H292 cells were cultured in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml penicillin and 100 U/ml streptomycin. HFL1 cells were cultured in DMEM-F12 supplemented with 10% FBS, 50 μg/ml penicillin, and 50 U/ml streptomycin. Human bronchial epithelial cells (Beas-2B) were grown in DMEM-F12 supplemented with 5% FBS, 15 mM HEPES, 100 μg/ml penicillin, and 100 U/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 and 3% O_2 controlled incubator. HFL-1 cells were grown to 80–90% confluence and replaced with 0.5% FBS 24 hrs prior to treatment. HFL-1 was treated with the following e-liquids: Propylene glycol, Glycerin, Vape Dudes (Classic tobacco with or without nicotine), Vape Dudes (Cinnamon roll without nicotine), Vape Dudes (Grape vape without nicotine), Ecto

Table 1. ENDS e-liquids their trade name, flavor, and manufacturer information obtained from local retailers used in this study.

Trade Name	Flavor	Form	Manufacturer
Blu	Classic Tobacco	Cartomizer	Lorillard
Blu	Magnificent Menthol	Cartomizer	Lorillard
Drip	Berry Intense	e-Liquid	Vaporotics
Drip	Melon Mania	e-Liquid	Vaporotics
Drip	Peaches 'n Cream	e-Liquid	Vaporotics
Drip	Pineapple Express	e-Liquid	Vaporotics
Ecto	American Tobacco	e-Liquid	Ecto
Encore	Tobacco	e-Liquid	Encore Vapor Inc.
Roc Juice	Tobacco	e-Liquid	Roc Juice Inc.
Roc Juice	Coconut	e-Liquid	Roc Juice Inc.
Upstate Vape	Mountain Dew	e-Liquid	Upstate Vape
Upstate Vape	Marbo	e-Liquid	Upstate Vape
Upstate Vape	9x Tobacco	e-Liquid	Upstate Vape
Vapor Drops	AMP	e-Liquid	-
Vapor Drops	Very Berry	e-Liquid	-
Vapor Drops	Tobacco	e-Liquid	-
Vape Dudes	Cinnamon Roll	e-Liquid	Vape Dudes
Vape Dudes	Classic Tobacco	e-Liquid	Vape Dudes
Vape Dudes	Cotton Candy	e-Liquid	Vape Dudes
Vape Dudes	Grape Vape	e-Liquid	Vape Dudes
Vape Dudes	Strawberry Fields	e-Liquid	Vape Dudes
Vape Dudes	Strawberry Zing	e-Liquid	Vape Dudes

doi:10.1371/journal.pone.0116732.t001

(American tobacco with or without nicotine) and other e-liquids (Table 1) for 24 hrs and then examined for morphological changes by phase-contrast microscopy at 20x magnification.

Preparation of aqueous cigarette smoke extract

Research grade cigarettes 3R4F were obtained from the Kentucky Tobacco Research and Development Center at the University of Kentucky (Lexington, KY). Cigarette smoke extract (CSE) was prepared by bubbling smoke from one cigarette into 10 ml serum-free media at a rate of one cigarette/min as described previously [25–27]. The CSE solution was then filter sterilized with a 0.45 μm syringe filter. CSE preparation was standardized by measuring the absorbance (OD: 1.00 ± 0.05) at a wavelength of 320 nm. The pattern of absorbance (spectrogram) observed at 320 nm showed very little variation between different preparations of CSE. CSE was freshly prepared for each experiment and diluted with culture media supplemented with 10% FBS immediately before use. For CSE treatments, HFL-1 cells were grown to 80–90% confluence and replaced with 0.5% FBS in DMEM (supplemented with 50 μg/ml penicillin, and 50 U/ml streptomycin) 24 hrs prior to CSE treatment at 37°C in a humidified atmosphere containing 5% CO_2 and 3% O_2 controlled incubator.

Air-liquid interface cell culture and exposure

H292 lung epithelial cells (ATCC) were grown at 37°C in a humidified atmosphere containing 5% CO_2, and 3% O_2 controlled incubator to 80–90% confluence in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml. streptomycin. Human bronchial epithelial cells (Beas-2B) were grown in DMEM-F12 supplemented with 5% FBS, 15

mM HEPES, 100 μg/ml penicillin, and 100 U/ml streptomycin. Cells were then sub-cultured into porous transwells (0.4 micron, Corning, Corning NY) at a density of 200,000 cells/transwell in 6-well plates. Transwell cultures were then placed into air-liquid interface exposure chamber [28–31]. Before placement into exposure chamber, cells were minimally overlaid with approximately 200 μl of RPMI media to prevent drying. Media is continuously exchanged through the sealed chambers ports via peristaltic pump while in contact with the porous bottom of the transwell. Using tubing connected to a lab pump (FMI, Syosset, NY). Blu e-cig aerosol (Classic tobacco flavor containing 16 mg nicotine) using a CSM-SSM machine (CH-Technologies Inc.) was drawn into the chamber every 30 seconds with a 4 second pulse [23] for different time durations 5, 10, and 15 minutes respectively. No treatment control/air group H292 cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and 3% O_2 controlled incubator for 16 hrs after Blu e-cig aerosol exposure to condition media.

Cell viability and flow cytometry

HFL-1 cells were cultured at 37°C, in a humidified atmosphere containing 5% CO_2, and 3% O_2 controlled incubator either in a 24-well plates (2.0 cm^2) or 6-well plates (9.6 cm^2) using appropriate growth media as described earlier. When the cells were about 80–90% confluent, fresh media was replaced with 0.5% FBS in DMEM (supplemented with 50 mg/ml penicillin, and 50 U/ml streptomycin) with or without e-liquids or CSE. Cell viability was measured after 24 hrs treatment by acridine orange/propidium iodide (AO/PI) staining using Cellometer 2000 (Nexcelom Bioscience, Lawrence MA). Beas-2B cells were trypsinized following 15 min exposure to Blu e-cig vapor in air-liquid interface chamber as described earlier. Following e-cig aerosol exposure, cells were washed in PBS, and analyzed on BD-LSRII system. An increase in cellular fluorescence after e-cig aerosol exposure was detected using a Violet B 405 nm laser and 440/40 band-pass filter. Data was compiled on FlowJo V. 10.

E-cigarette aerosol mouse exposure

Eight weeks old C57BL/6J mice were housed in the Inhalation Core Facility at the University of Rochester before being exposed to room air or e-cig aerosol exposure which was adapted as described previously for conventional cigarettes [32,33]. Blu e-cig (Classic tobacco flavor containing 16 mg nicotine) were used to generate the aerosols by a Teague smoking machine (Model TE-10, Teague Enterprises, Woodland, CA) at a concentration of approximately 200 mg/m^3 TPM. Mice received 5 h exposures per day for 3 successive days. A new TE-10 Teague machine was modified and dedicated only for ENDS/e-cig aerosol exposures *in vivo*. All animal protocols described in this study were approved by the University Committee on Animal Research Committee of the University of Rochester.

Bronchoalveolar lavage

Mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (100 mg/kg; Abbott Laboratories, Abbott Park, IL) and then sacrificed by exsanguination in two different batches one immediately after 5 hrs e-cig exposure on the 3rd day and another batch 24 hrs after last e-cig exposure. The lungs were lavaged three times with 0.6 ml of saline via a cannula inserted into the trachea. The aliquots were combined and centrifuged, and the bronchoalveolar lavage (BAL) fluid stored at -80°C for cytokine/chemokine analysis and the cell pellet was resuspended in saline. The cells were stained with AO/PI stain and the total cell number was counted using Cellometer 2000 (Nexcelom Bioscience, Lawrence MA). Cytospin slides (Thermo Shandon, Pittsburgh, PA) were prepared using 50,000 cells per slide, and differential

cell counts (~500 cells/slide) were performed on cytospin-prepared slides stained with Diff-Quik (Siemens, DE).

Pro-inflammatory mediators analysis

Following 24 hrs humectant/e-liquid treatment, conditioned media was collected and stored at -80°C for measuring pro-inflammatory mediators. IL-8 and IL-6 levels were measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (Life Technologies, Carlsbad, CA). Pro-inflammatory mediators in bronchoalveloar lavage fluid (BALF) collected from room air and e-cig aerosol exposed mice 24 hrs after the last Blu e-cig exposure were measured using ELISA according to the manufacturer's instructions (MCP-1 and IL-6). Various cytokines/chemokines from BAL fluid were measured by the Luminex Flexmap3D system (Austin, TX) using Milliplex mouse cytokine/chemokine magnetic bead panel for Luminex platform according to manufacturer's instructions (Billerica, MA).

Cotinine assay

Levels of cotinine in mouse plasma samples collected immediately after the 3rd day Blu e-cig aerosol exposure (5 hrs) was measured by ELISA according to manufacturer's instructions (Abnova, Taipei, TW).

Glutathione and glutathione disulfide measurements

Total and oxidized (disulfide) glutathione levels measured in mouse lung harvested immediately after the 5 hrs Blu e-cig aerosol exposure (3rd day) as described previously [34]. In brief, the concentration of total glutathione in the supernatant of lung homogenates was determined by comparison with the colorimetric rate of DTNB reduction by known standard concentrations of reduced glutathione (GSH). For determining the concentration of oxidized glutathione/glutathione disulfide (GSSG), lung homogenates were combined with 2% of 2-vinylpyridine (VP) to derivatize (masking) endogenous GSH. Excess VP is neutralized by triethanolamine so that in the subsequent reaction, glutathione reductase is able to recycle endogenous GSSG back into underivatized GSH. GSSG levels are then indirectly measured by DTNB reduction by newly reduced GSH, which was produced from endogenous GSSG *in vitro*. Results were expressed as the nmol of total glutathione and GSSG per mg protein as well as total glutathione/GSSG ratio. All sample homogenates were prepared using RIPA buffer and underwent multiple freeze thaw cycles prior to measuring glutathione levels.

Statistical analysis

Statistical analysis of significance was calculated using unpaired Student's *t*-test. Probability of significance compared to control was based on 2-tail *t*-tests and indicated in figure legends. The results are shown as the mean ± SD unless otherwise indicated. A value of $P < 0.05$ is considered as statistically significant.

Results

ENDS/e-cigarette OX/ROS generation

OX/ROS produced by ENDS/e-cigs were detected by drawing the aerosols through a fluorescein derived dye (DCFH solution) using an air flow pump (see Materials and Methods). The oxidized form of DCFH (DCF) emits green fluorescence following excitation at 490 nm indicating OX/ROS or ROS activity. In both cell and cell-free systems, DCFH serves as a semi-quantitative

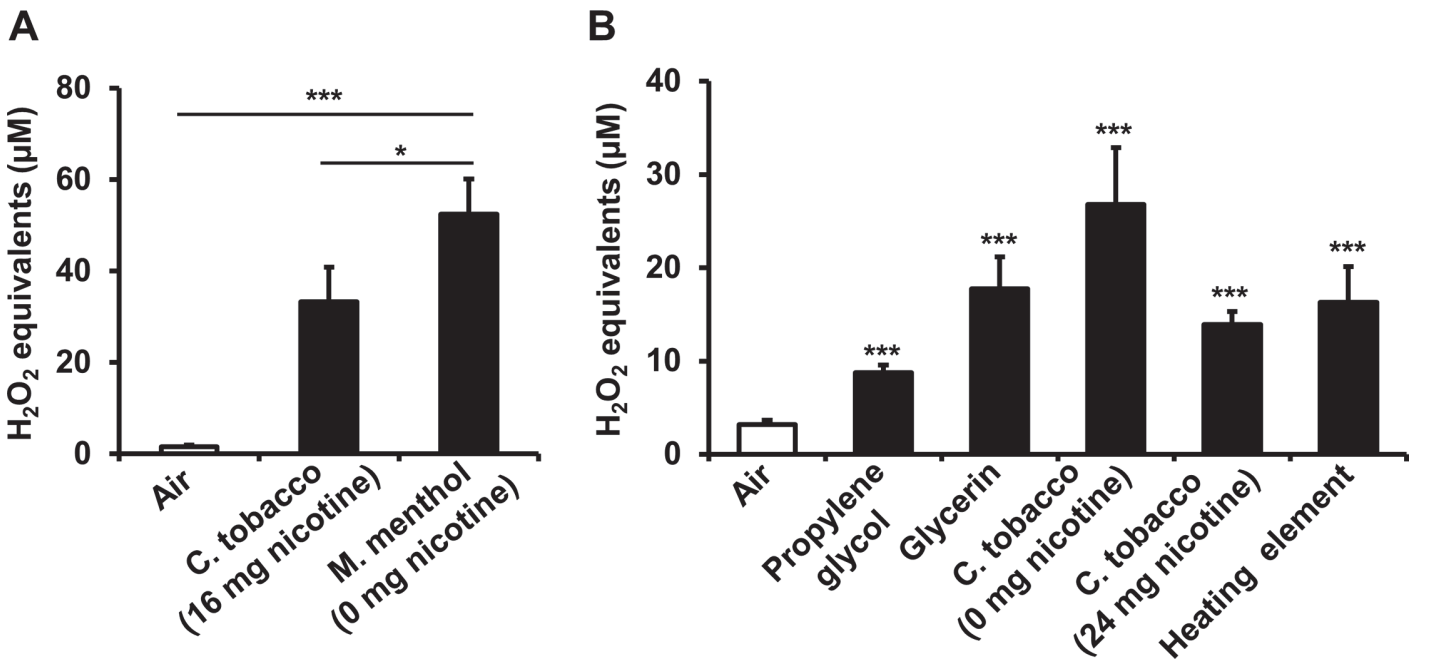

Fig 2. OX/ROS in ENDS vapor. Aerosols or air-sham control drawn through DCFH OX/ROS indicator solution. (A) Blu e-cigarette cartomizers; Classic tobacco or Magnificent menthol flavor e-cigs. Data are shown as mean ± SD (n = 3/group).* $P < 0.05$, *** $P < 0.001$ compared to air-sham control (B) eGo refillable vaporizer. Humectants; propylene glycol and glycerin. Commercial e-liquid refills; Vape Dudes Classic tobacco flavor. Data are shown as mean ± SEM (air, n = 15; propylene glycol, n = 23; glycerin, n = 21; Vape Dudes C. tobacco 0 mg nicotine, n = 7; Vape Dudes C. tobacco 24 mg nicotine, n = 3; Heating element, n = 9). *** $P < 0.001$ compared to air-sham control.

doi:10.1371/journal.pone.0116732.g002

indicator for presence of reactive OX/ROS and has been used previously to measure nanoparticle mediated oxidation in cell free systems [35].

Detection for the presence of OX/ROS in Blu e-cig vapor was performed using two different flavored Blu e-cig cartomizers (Classic Tobacco or Magnificent Menthol) (**Fig. 1A**). Each cartomizer varies in nicotine content (0 mg and 24 mg) and both were included to assess if aerosols produced within the cartomizers give rise to major differences in DCF fluorescence intensity after they were drawn through DCFH solution. Aerosols drawn through DCFH produced from the classic tobacco flavor cartomizer (16 mg of nicotine) resulted in increased H_2O_2 μM equivalents (equivalent to DCF fluorescence intensity units) as compared to air-sham group (**Fig. 2A**). The levels of H_2O_2 μM equivalents from the menthol cartomizer aerosols were also significantly increased (**Fig. 2A**). Comparison of OX/ROS levels between both of the cartomizer aerosols showed that the one containing nicotine resulted in significantly reduced levels of H_2O_2 μM equivalents (**Fig. 2A**).

Next, we exchanged the Blu e-cigs with a different type of popular refillable ENDS to test for OX/ROS reactivity in ENDS aerosol. The eGO Vision Spinner with a 2.2 ohm "wicked" heating element and clearomizer chamber capable of holding 4.5 ml of e-liquid is noticeably larger than the Blu e-cig (**Fig. 1A**) and produces aerosols in a similar fashion. The e-liquids, sold in a plethora of flavors are primarily comprised of humectants propylene glycol and glycerin [36]. Propylene glycol was filled into the clearomizer and aerosols produced from the refillable ENDS elicited an increase in H_2O_2 μM equivalents as compared to air-sham group (**Fig. 2B**). Similarly, aerosols produced exclusively from glycerin also reacted with DCFH leading to significantly increased H_2O_2 μM equivalent levels (**Fig. 2B**). Two of the commercially available e-liquids (Vape Dudes and Classic tobacco flavor) were also tested for OX/ROS reactivity using the refillable ENDS device. One of these samples contains 0 mg nicotine and the other contains

24 mg nicotine in addition to undisclosed mixtures of propylene glycol, glycerin, and flavor additives. Both the non-nicotine and nicotine containing commercially available e-liquids produced aerosols that resulted in increased H_2O_2 μM equivalents levels (Fig. 2B).

These results suggest OX/ROS are emanating from the e-cigs/e-liquids and are associated with the aerosols that are drawn through the DCFH indicator, and nicotine was not likely a sole contributing factor in increased OX/ROS reactivity.

Source of OX/ROS generation from ENDS/e-cigarettes

It was not clear if the OX/ROS we detected were exclusive to the aerosols of the ENDS or if they might emanate from another source within the device. We determined that there are two possible sources of OX/ROS that are generated by the refillable ENDS device. One of the sources of OX/ROS appears to be the heating element since there is an increase in OX/ROS when the heating element is activated without e-liquid filled into the clearomizer chamber (Fig. 2B). In this case, air is drawn through the device by the pump as the ENDS device is activated, however, there was no visible sign of aerosol being produced. From this data, we conclude it is possible to generate OX/ROS from ENDS independent of e-liquid vaporization.

OX/ROS detection in ENDS aerosols overall yielded a rather broad range of measurements for DCF fluorescence including what we defined as "high range" values. Attaining a high range value measurement required a 1:10 dilution in pristine DCFH solution to extrapolate their final values. High range aerosol-DCF fluorescence values, including the values for OX/ROS detected in ambient air flow from activating the heating element without e-liquids, were partitioned and compiled together (Table 2).

To validate whether or not OX/ROS reactivity emanating from the refillable ENDS device occurs either by vaporizing e-liquids/humectants, or activating the heating element without e-liquids/humectants, we hypothesized that the state of the heating element (new versus multi-use) affects the capacity for OX/ROS to be generated by the refillable ENDS. We first cleaned and refilled the removable clearomizer chambers with 2.0 ml of either propylene glycol, glycerin, or a commercial refill e-liquid (Vape Dudes Classic tobacco, 0 mg nicotine). A new set of new replacement 2.2 ohm heating elements was obtained from a local merchant that sells e-cigs and accessories and three of the heating elements that were used a number of times in previous experiments (over 50 times use, exact number of uses unknown) retained. For each e-liquid/humectant, two repeat trials were conducted with a pre-used heating element, drawing aerosols into DCFH solution in exactly the same manner and timing as for our previous DCFH experiments. Aerosols for each e-liquid/humectant drawn through DCFH indicate the presence of OX/ROS compared to air-sham control which did not result in any appreciable level of OX/ROS reactivity (Table 3, **Experiment 1**). Next, in order to determine if replacing the pre-used heating element with a new one is able to achieve "high range" range DCF fluorescence, the same sample of e-liquid/humectant in the clearomizer from Trial 1 and Trial 2 was retained. Each DCF fluorescence value obtained after installing the new heating element required 1:10 dilutions in DCFH solution (Table 3, **Experiment 1**). These results suggest that the state of heating element after activation affects the generation of OX/ROS by the refillable ENDS.

We further confirmed that the state of the heating element affects OX/ROS generation by installing a new heating element and activating it independently of e-liquids (empty clearomizer) for three trials. As the state of the heating element transitions from new to multi-used between trials, it's generation of OX/ROS approached air-sham control level of DCF fluorescence (Table 3, **Experiment 2, New, 2nd use, and 3rd use)**. We then hypothesized that if the vaporization process of the e-liquids is also a source of OX/ROS generation, then adding e-liquid for the 4th use of the used heating element will lead to a spike in DCF fluorescence after the

Table 2. DCF fluorescence values obtained for refillable ENDS aerosols or ambient air alone drawn through DCFH in cell-free ROS assay.

Humectants	H_2O_2 equivalents (μM) †
Propylene glycol	120.7
	129.0
	127.3
Mean ± SEM ‡	125.7 ± 2.5*** (93.0) #
Glycerin	211.5
	305.2
	312.4
	360.7
	200.2
	145.6
Mean ± SEM ‡	255.9 ± 33.6*** (93.1) #
Propylene glycol: Glycerin (50:50)	412.5
	360.5
	146.8
Mean ± SEM ‡	306.6 ± 81.3*** (97.0) #
Classic tobacco (0 mg nicotine)	248
	113.6
	103.2
	134.0
	131.0
Mean ± SEM ‡	146.0 ± 26.1*** (81.6) #
Classic tobacco (24 mg nicotine)	327.7
	103.8
	60.4
Mean ± SEM ‡	164.0 ± 82.8*** (91.5) #
Pre-used heating element (without e-liquid)	250.5
	192.0
	84.2
Mean ± SEM ‡	175.6 ± 48.7*** (90.7) #
Air-Sham (control)	3.89
	4.6
	4.0
	2.1
	1.5
	1.6
	1.4
	1.2
	1.2
	2.5
	4.6
	2.8
	4.8
	4.9
	7.1

(*Continued*)

Table 2. (*Continued*)

Humectants	H_2O_2 equivalents (μM) †
Mean ± SEM ‡	3.2 ± 0.46

DCF fluorescence values (high range) from refillable ENDS aerosols or ambient air flowing through activated ENDS heating element. Each fluorometer reading indicates that oxidation to DCF is diluted 1 to 10 with pristine DCFH solution to attain fluorometer measurements within calibration range of the high standard (50 μM H_2O_2).

† Each value shown in H_2O_2 equivalents (μM) for humectants represents individual trials analyzed by cell-free ROS assay.

‡ Compared with Air-Sham (control) and from values shown in Fig. 2B.

♯ The percentage of change from non-high range to high range values is based on values obtained from data quantitated in Fig. 2B and compared to the high range values in Table 2.

***P<0.001 vs Air-Sham (control) and from values shown in Fig. 2B.

doi:10.1371/journal.pone.0116732.t002

Table 3. State of the refillable ENDS heating element and its influence over successive use to generate OX/ROS in a cell-free ROS assay.

		State of the heating element		
Experiment 1	**Pre-used**		**New**	
Humectants	**Trial 1**	**Trial 2**	**Single use only**	
Propylene glycol	15.32	13.06	127.23	
Glycerin	20.65	34.97	305.2	
Consumer refill	47.55	37.42	133.97	
Air (sham)	1.19	2.08	1.17	
Experiment 2	**New**	**2nd use**	**3rd use**	**4th use**
Powered	33.28	8.99	5.68	135.6†
Air (sham)	1.60	1.50	1.39	-
Experiment 3		**Pre-used**		
	Clearomizer filled with e-liquid		**Emptied clearomizer with wicked e-liquid**	
Humectant	**Trial 1**	**Trail 2**	**Trail 3**	**Trail 4**
Consumer refill	47.55	37.42	192.40	250.50

Each flourometric value shown in this table represents the H_2O_2 equivalents (μM) measured after aerosols produced from different humectants/Vape dudes e-liquids (classic tobacco, 0 mg nicotine) or ambient air moving through the activated device are drawn through DCFH solution to test the role of heating element state by cell-free ROS assay. DCF fluorescence values less than 3.2 H_2O_2 equivalents (Average air-sham control values determined in **Fig. 2B**) were not considered to contain oxidants.

† Fluorometric value shown in 4th use is after direct addition of e-liquid to the wick analyzed by cell-free ROS assay.

Experiment 1: Clearomizer chamber is filled with ~2.0 mL humectant or e-liquid. A previously used heating element is installed into the device for Trial 1 and 2. The third trial is carried out after exchanging the used heating elements for new ones (single use).

Experiment 2: A never before used heating element is installed into the refillable ENDS. The ENDS is activated and ambient air is drawn through the device and then into DCFH solution. The experiment is repeated for successive 3 trials using that same heating element. After the third trial (heating element 3rd use), 2 drops of e-fluid is "dripped" onto the wick and allowed to absorb. Aerosols are then produced from the "dripped" e-liquid and drawn into DCFH (4th use).

Experiment 3: After loading the clearomizer with ~ 2.0 of e-liquid, a heating element used from previous experiments is installed into the device and the e-liquid aerosols that it produces are drawn into DCFH for 2 trials. For trials 3 and 4, the clearomizer chamber is completely emptied and the used heating element wick allowed to retain absorbed e-liquid before producing aerosols and drawing them into DCFH.

doi:10.1371/journal.pone.0116732.t003

aerosols are drawn through DCFH solution. Rather than filling the clearomizer with e-liquid, a single drop of e-fluid was absorbed into the heating element wick. The 4th use heating element trial was completed after 5 minutes (half number of puffs) rather than the usual 10 minutes for all other trials. The resultant DCF fluorescence value required a 1:10 dilution in DCFH solution (**Table 3, Experiment 2, 4th use**). Overall, these results suggest that there are at least two possible sources of OX/ROS released from ENDS, 1) from activation of the heating element, and 2) the process of vaporizing e-liquids.

ENDS "dripping" technique and OX/ROS generation

The use of a refillable clearomizer chamber for ENDS is typical for securing e-liquids while consumers inhale their aerosols. An emerging trend abandons use of the clearomizer and replaces it for an inhalation tip that does not hold e-fluid. The "drip tip" allow consumers to "drip" e-liquid directly onto the heating element wick in the same manner as we applied e-liquid to the heating element for the 4th use (**Table 3, Experiment 2**). To determine whether or not the clearomizer filled with e-liquid versus dripping the e-liquid onto the heating element wick leads to high range fluorescence values (requires 1:10 dilution in DCFH solution), a pre-used functioning heating element was installed into the refillable ENDS. In the first two trials, aerosols produced by e-liquid filled into the clearomizer resulted in detection of OX/ROS and the DCF fluorescence values attained did not require 1:10 dilutions (**Table 3, Experiment 3**). In contrast, aerosols produced in trials 3 and 4 were carried out by "dripping" small amounts of e-liquid sufficient to absorb into the wick without any liquid placed into the clearomizer. Aerosols produced in this manner resulted in high range DCF fluorescence values which required 1:10 dilutions in DCFH solution to attain fluorometer readings (**Table 3, Experiment 3**). These results suggest that the emerging trend of "dripping" e-liquids to produce ENDS aerosols delivers a larger dose of OX/ROS to consumers.

Reactivity of commercial e-fluids with DCFH

A variety of locally purchased commercially available e-liquids differing in nicotine content and or flavor were reacted with the DCFH solution directly. Water and the purified humectants propylene glycol and glycerin showed no appreciable indication of reactivity with DCFH. All of the flavored e-liquids exhibited various DCFH reactivity (**Table 4**). When e-liquid DCF fluorescence values from Table 4 were compared by nicotine content irrespective of brand or flavor, the nicotine containing e-liquids exhibited significantly less DCFH reactivity (**Fig. 3A**). Table 4 depicting e-liquids that contained non-tobacco flavor additives (dessert, fruit, and candy) where on average significantly more reactive with DCFH than e-liquids recreating tobacco flavors (**Fig. 3B**), suggesting more oxidative reactivity and injurious response by flavored e-liquids.

Human lung fibroblasts exhibited stress and morphological change in response to e-liquids/humectants

The effect of exposing lung cells directly to e-liquids is not known. Since OX/ROS reactivity is associated with ENDS e-liquids in the cell-free conditions, we asked whether or not commercially available e-liquids or purified humectants may induce any obvious morphological signs of cell stress in normal human primary lung cells. After 24 hrs, near to confluent fibroblasts treated in either 1% or 5% propylene glycol, glycerin, or tobacco flavored commercial e-liquid (Ecto) exhibited various morphological alterations (**Fig. 4A**). Fibroblasts cultured with e-liquid or CSE exhibited a reduction in the number of cells per count area (**Fig. 4B**). Many of the treated cells were enlarged and vacuolarized, and this effect was greater in CSE treated cells and cells treated with 5% e-liquids (**Fig. 4A**). Compared to control cells, e-liquid and CSE treated

Table 4. DCF fluorescence of refillable e-liquids with different flavors and nicotine concentrations after addition of DCFH solution analyzed by a cell-free ROS assay.

Vape drops †			Vape dudes †		
Flavors	**Nicotine conc. (mg)**	**DCF (FIU)**	**Flavors**	**Nicotine conc. (mg)**	**DCF (FIU)**
Tobacco	0	63.15	Classic tobacco	0	95.82
Tobacco	6	30.92	Classic tobacco	24	54.48
Tobacco	11	16.47	Cinnamon roll	0	82.8
Tobacco	18	16.33	Grape vape	0	75
Tobacco	24	18.64	Cotton candy	0	94.08
Very berry	0	101.3	Strawberry zing	0	101.3
AMP	0	83.53	Strawberry fields	0	61.71
Ecto †			**Drip †**		
American tobacco	0	85.69	Peaches'n cream	0	95.52
American tobacco	12	81.07	Berry intense	0	88.87
American tobacco	18	80.2	Pineapple express	0	99.28
American tobacco	24	81.65	Melon mania	0	94.94
Upstate vape †			**Encore †**		
9x Tobacco	0	92.2	Tobacco	16	12.43
9x Tobacco	11	47.4	Tobacco	24	14.16
9x Tobacco	18	35.4	**Roc juice †**		
9x Tobacco	24	31.21	Tobacco	0	101.2
Marbo	0	43.79	Tobacco	6	20.6
Marbo	6	49.71	Tobacco	18	18.79
Marbo	11	37.14	Tobacco	24	20.52
Marbo	18	24.57	Coconut	24	16.62
Marbo	24	53.32			
Mountain dew	18	79.62			

† 167 μl e-liquid added to final volume of 5 ml DCFH solution, equivalent to volume of H_2O_2 added to attain a fluorometric value of approximately 50 FIU. DCF fluorescence for 50 μM H_2O_2 standard (50.02), humectant polyethylene glycol (0.43), humectant glycerin (1.3) and vehicle/water (0.03).

doi:10.1371/journal.pone.0116732.t004

cells showed hetero-morphological structures (enlarged cells and spindle formation) in e-liquid treated cells. Commercially available e-liquid added to cells at 1% concentration without nicotine, displayed similar morphological alterations to that of 1% propylene glycol. In contrast, fibroblasts cultured in 1% e-liquid that do contain nicotine, resulted in more profound morphological changes that resemble cells treated with 1% CSE [37]. There was also considerable cell overlap and mixed directional orientation throughout the image field for cells treated with 1% e-liquid containing nicotine. Vacuolization and cell enlargement following treatment with 5% e-liquid containing nicotine was most similar to fibroblasts treated with 1% CSE. There is also almost complete loss of the fusiform structure typical of fibroblasts in culture (control). Both CSE and e-liquid treated cells also showed a prevalence of larger adhered circular cells, noticeable due to the halo effect inherent in phase-contrast microscopy. These results suggest when e-liquids are applied directly to lung fibroblasts at these concentrations, there were signs of cell stress and other phenotypic abnormalities that are further exacerbated by nicotine.

ENDS e-liquids/humectants and cell viability

To assess how e-liquids/humectants affect cell viability relative to CSE treatment, normal human lung fibroblasts were first cultured in 35 mm dishes and grown to 90% confluence. The

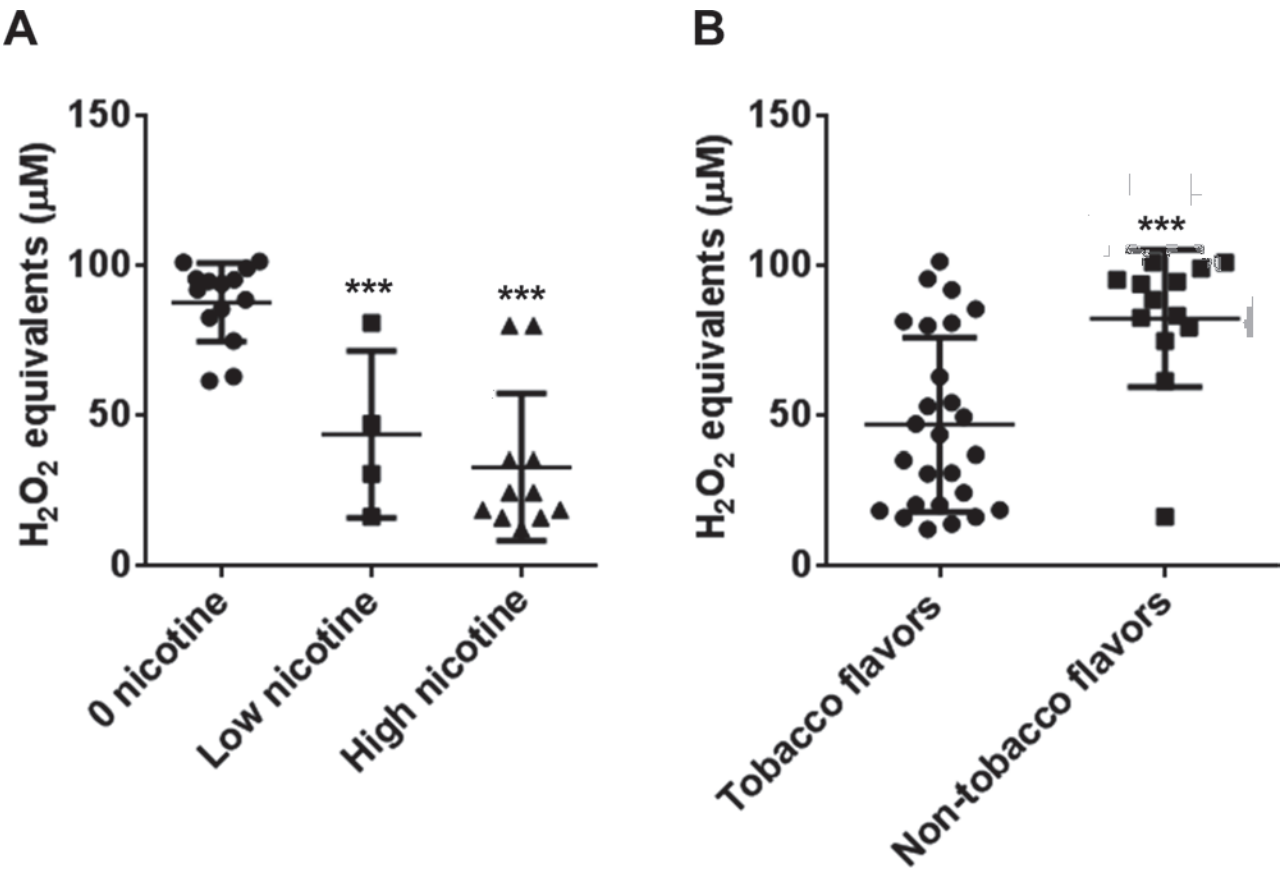

Fig 3. E-liquid reactivity with DCFH exhibits differences between nicotine content and flavor additives. (A) Commercially available e-liquids with different nicotine content. No nicotine (0 mg), low nicotine (6–12 mg) and high nicotine (16–24 mg). Data are shown as mean ± SD. *** $P < 0.001$. (B) Comparison of commercially available e-liquids, tobacco flavors (Tobacco, American tobacco, Classic tobacco 9x Tobacco, Marbo) versus non-tobacco flavors (Very berry, AMP, Mountain dew, Cinnamon roll, Grape vape, Cotton candy, Strawberry zing, Strawberry fields, Peaches n cream, Berry intense, Pineapple express, Melon mania, and Coconut). Data are shown as mean ± SD of n = 3, *** $P < 0.001$. Y-axis equal to DCF fluorescence Intensity Units (FIU).

doi:10.1371/journal.pone.0116732.g003

cells were then shifted to medium containing 2.5% propylene glycol, glycerin, commercial e-liquids (0 mg or 24 mg of nicotine), or CSE (1%) and measured for viability after 24 hrs. A treatment condition of 1% CSE was also included, in which we have been able to maintain high density normal human lung fibroblasts for over 24 hrs without a significant cell loss/viability [38].

Lung fibroblast viability following treatments with 2.5% propylene glycol, glycerin, or commercial e-liquids was not significantly different than control after 24 hrs (Control; 90.53 ± 5.34, Propylene Glycol; 88.40 ± 2.99, Glycerin; 91.97 ± 6.23, Ecto American tobacco flavor 0 mg nicotine; 92.7 ± 2.55, Ecto American tobacco flavor 24 mg nicotine; 78.57 ± 6.67, % viability in means ± SD, $p > 0.05$%). As expected, 2.5% CSE treatment caused significant cell death, leading to less than 20% viability after the 24 hrs (CSE; 12.7 ± 4.73, % viability in means ± SD, $p < 0.001$). Conversely, viability for fibroblasts treated with 1% CSE was not significantly different than control, similarly to cell treatments with 2.5% e-liquid/humectant (CSE; 89.4 ± 5.86% viability in means ± SD). Therefore, the lung fibroblasts were more sensitive to CSE than e-liquids/humectants which did not exhibit an apparent effect on cell viability when treated at a higher concentration than CSE. However, in our initial assessments, we observed a global decrease in HFL-1 cell viability when the cells were cultured within smaller growth areas

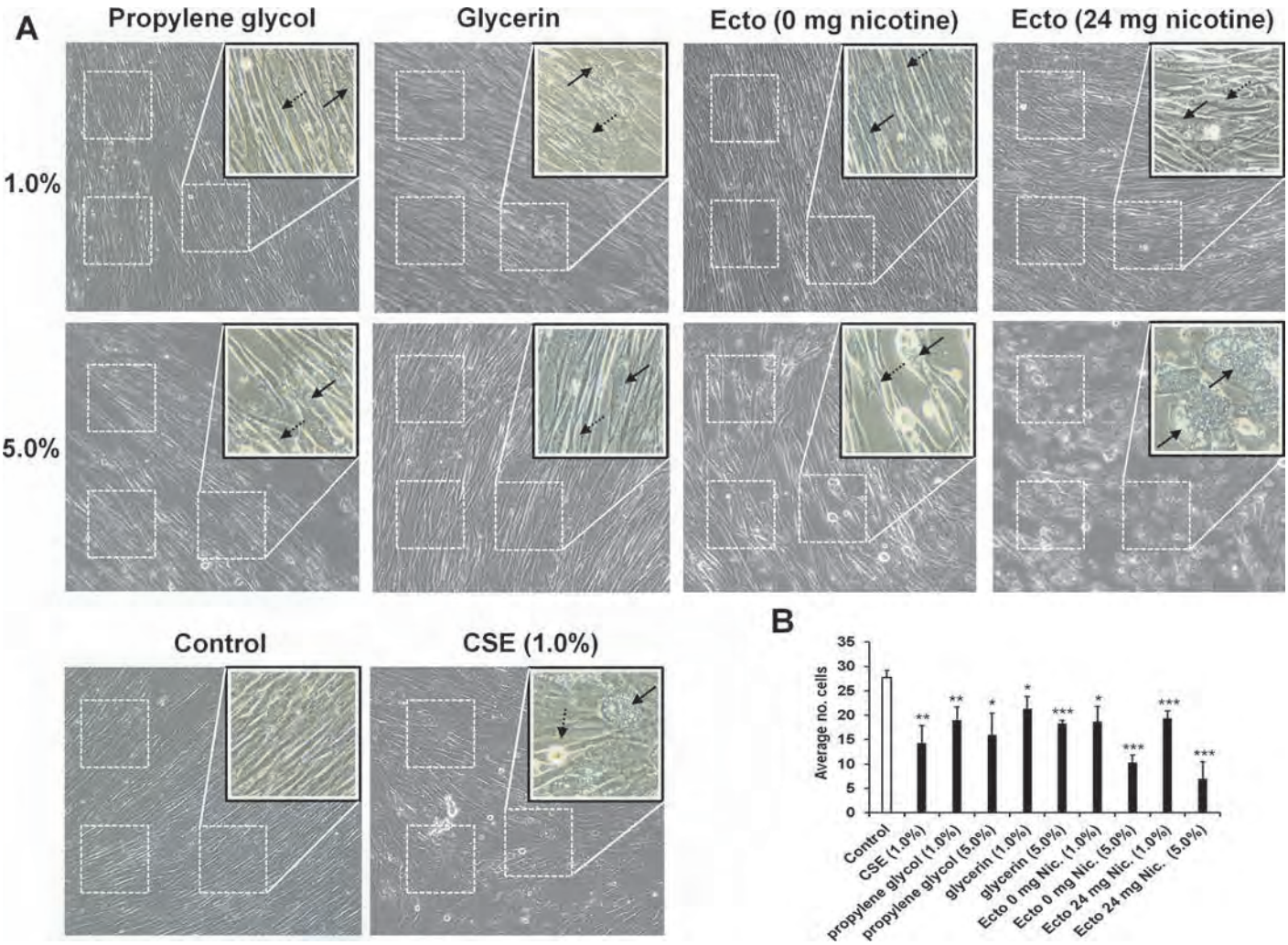

Fig 4. Addition of e-liquids to cell culture media induces morphological changes in human lung fibroblasts. (A) HFL-1 cells grown to 95% confluence were treated with the following e-liquids; propylene glycol, glycerin, or Ecto American tobacco flavor for 24 hrs and then examined for morphological changes by phase-contrast microscopy. Treatment of HFL-1 with 1.0% CSE for 24 hrs included for comparison. Images captured at 20x magnification. Embedded images show expansion of defined area of monolayer as demarcated by dashed boxes. Representative images are shown (n = 3). Enlarged vacuolarized cells in expansion area or large circularized cells (solid arrow), and areas of lost cell-cell connection next to spindle formations (dashed arrow) within defined area vs control. (B) Average number of cells counted adjacently across a single diagonal of 3 defined areas placed randomly (dashed boxes within images). The direction of diagonal cell counts is based on cell orientation in each image. Data are shown as mean ± SD. *$P < 0.05$; **$P < 0.01$; and *** $P < 0.001$ as compared to untreated control culture in growth media.

doi:10.1371/journal.pone.0116732.g004

(15.5 mm dishes). E-liquid concentrations starting at 0.5% decreased cell viability below 50% for three of the commercial e-liquid brands after 24 hrs (Table 5). The decrease in cell viability broadened after increasing the e-liquid concentration to 5% and then 10% in the smaller culture dishes. Since cell viability after treatment with 2.5% e-liquid/humectant in larger culture areas was not reduced compared to control cells, the susceptibility to loss of cell viability by direct addition of e-liquids to culture media depends on size of the cell population.

ENDS e-liquid flavor additives mediate release of IL-8 in lung fibroblasts

Interleukin 8, a cytokine that functions as a chemoattractant for inflammatory leukocytes and is released from lung cells after exposure to cigarette smoke was measured in conditioned

Table 5. Effect of e-liquids on HFL-1 cell viability in small 24-well culture area after 24 hours.

E-liquid (%)	Nicotine concentration (mg)											
	0			12			18			24		
	UV	E	RJ	UV	E	RJ	UV	E	RJ	UV	E	RJ
0.5	85.9	70.2	40.5[Ŧ]	47.9[Ŧ]	31.3[Ŧ]	26.5[Ŧ]	82.9	89.4	24.6[Ŧ]	58.5	68.5	19.0[Ŧ]
5.0	42.6	33.0	16.4	21.5	54.0	29.7	25.0	36.8	18.0	16.5	10.9	13.1
10.0	20.7	1.3	27.1	1.4	0.0	36.4	1.2	0.8	1.0	0.0	0.0	0.0
	Mean ± SD											
0.5	65.5 ± 23.05			35.2 ± 11.2*			65.6 ± 35.7*			48.7 ± 26.2*		
5.0	30.7 ± 13.3			35.1 ± 16.9*			26.6 ± 9.5*			13.5 ± 2.82*		
10.0	16.4 ± 13.4			12.6 ± 20.6*			13.5 ± 2.8*			0.0 ± 0.0*		

HFL-1 control cells without any treatment showed 95.4% viability.

E-liquids (UV: Upstate vape; E: Ecto; and RJ: Roc juice) at concentrations were used in this study (0.5%, 5.0% and 10.0%) for measuring percentage viability in HFL-1 cells after 24 hrs treatment.

[Ŧ] values below 50% viability in 0.5% e-Liquid.

* NS compared to 0 mg nicotine

doi:10.1371/journal.pone.0116732.t005

media from normal human lung fibroblasts treated with 1% e-liquids/humectants or 0.5% CSE. Neither of the pure humectants (propylene glycol, glycerin) elicited significant increase in release of IL-8 compared to control group (15.9 ± 12.02 pg/ml) after 24 hour treatment (Fig. 5A). Of the four commercially available e-liquids (Vape Dudes), only cinnamon roll flavored e-liquid stimulated a significant increase in IL-8 secretion (458.14 ± 26.20 pg/ml). The tobacco flavored e-liquid containing 24 mg nicotine, although eliciting slightly higher levels of IL-8 secretion than control cells (18.60 ± 4.79 pg/ml), was not statistically significant. However, although IL-8 secretion by tobacco flavored e-liquid containing 0 mg of nicotine was also not significantly different from the control group, the tobacco flavored e-liquid containing 24 mg of nicotine yielded significantly higher IL-8 levels (18.59 ± 4.79 pg/ml) compared to tobacco flavored e-liquid containing 0 mg of nicotine (5.28 ± 4.03 pg/ml). This suggests that nicotine added to e-liquid had a striking effect on IL-8 secretion in lung fibroblasts. Treating cells with 0.5% CSE, significantly increased fibroblast IL-8 secretion (83.81 ± 8.99 pg/ml). Since cinnamon flavor e-liquid is capable of stimulating a significant increase in IL-8 secretion from lung fibroblasts, while other e-liquid flavors (tobacco and grape) do not, certain e-liquid flavor additives can stimulate an inflammatory response in cultured lung fibroblasts.

Human airway epithelial cells directly exposed to e-cigarettes vapor increase IL-8 and IL-6 secretion

Using an air-liquid interface culture system, human lung H292 epithelial cells were directly exposed to tobacco flavor Blu e-cig aerosols. IL-8 and IL-6 secretion measured at 16 hrs after air-liquid interface exposure for each exposure time period was significantly higher than air groups (Fig. 5B, D). The release of IL-6 into culture media also occurred in a dose-dependent manner in response to the aerosol exposures. IL-6 secreted following 10 minute exposures to e-cig aerosols were significantly higher than the 5 minute exposures (Fig. 5C). The IL-8 levels induced by air-liquid interface aerosols in H292 were all significantly increased compared to air group. However, they did not exhibit a dose-dependent effect over increasing exposure periods (Fig. 5B).

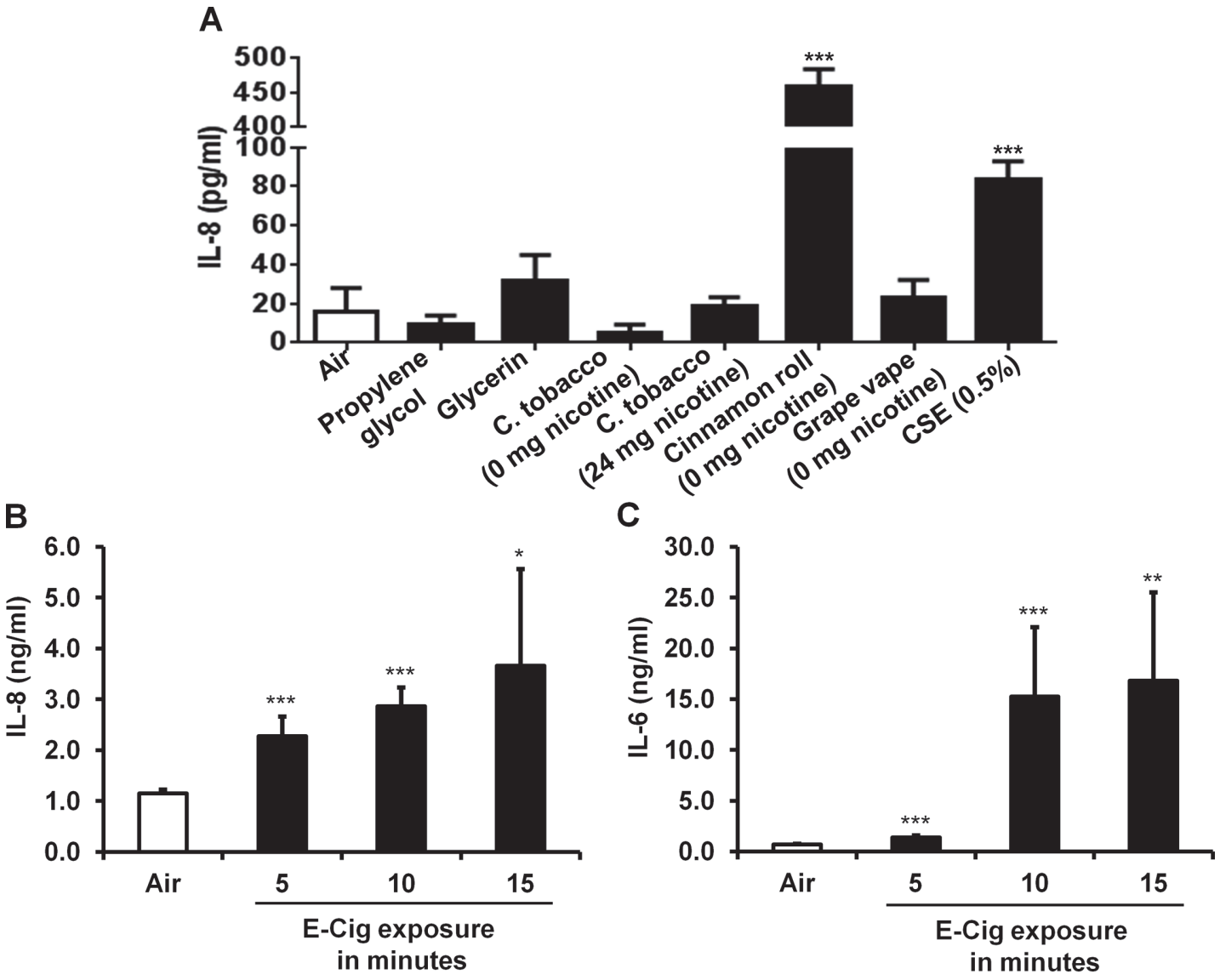

Fig 5. Inflammatory mediators secreted by human lung fibroblasts (HFL-1) treated with e-liquids/humectants and human epithelial airway cells (H292) treated by air-liquid interface with e-cigarette aerosols. (A) Levels of IL-8 release in conditioned media from HFL-1 cells treated for 24 hrs with 1% humectants or e-liquids or CSE were measured by ELISA. Data are shown as mean ± SD of n = 3. *** $P < 0.001$ compared to control cells maintained in media with 0.5% FBS. (B) H292 cells were exposed to Blu e-cigarette aerosols with a puff of 3–4 sec for 5, 10 and 15 min. After exposure, H292 cells were incubated at 37°C in 5% CO_2 incubator for 16 hrs and levels of IL-8, and (C) IL-6 release in conditioned media were measured by ELISA. Data are shown as mean ± SD. *$P < 0.05$; **$P < 0.01$; and *** $P < 0.001$ as compared to air group (cells maintained in incubator).

doi:10.1371/journal.pone.0116732.g005

Next, we observed evidence of a non-specific e-cig substance associated with its aerosol that could emit a fluorescent signature after aerosol deposition onto the cells in the air-liquid interface chamber that may be associated with oxidative and inflammatory responses. Beas-2B cells exposed for a 15 minute period (4 sec. puffs every 30 sec.) with Blu e-cig aerosols were harvested and analyzed by flow cytometry using 2 different colored lasers (488, and 405 nm). In cells exposed to e-cig aerosols, we detected a small but significant increase in fluorescence utilizing the 405 nm laser with 440/40 band pass filter (Fig. 6). This result alludes to the possibility that e-cig aerosol constituents can adhere to cell surfaces despite those surfaces being submerged under a thin layer (1–2 mm) of culture media, and become pro-oxidant and inflammatory.

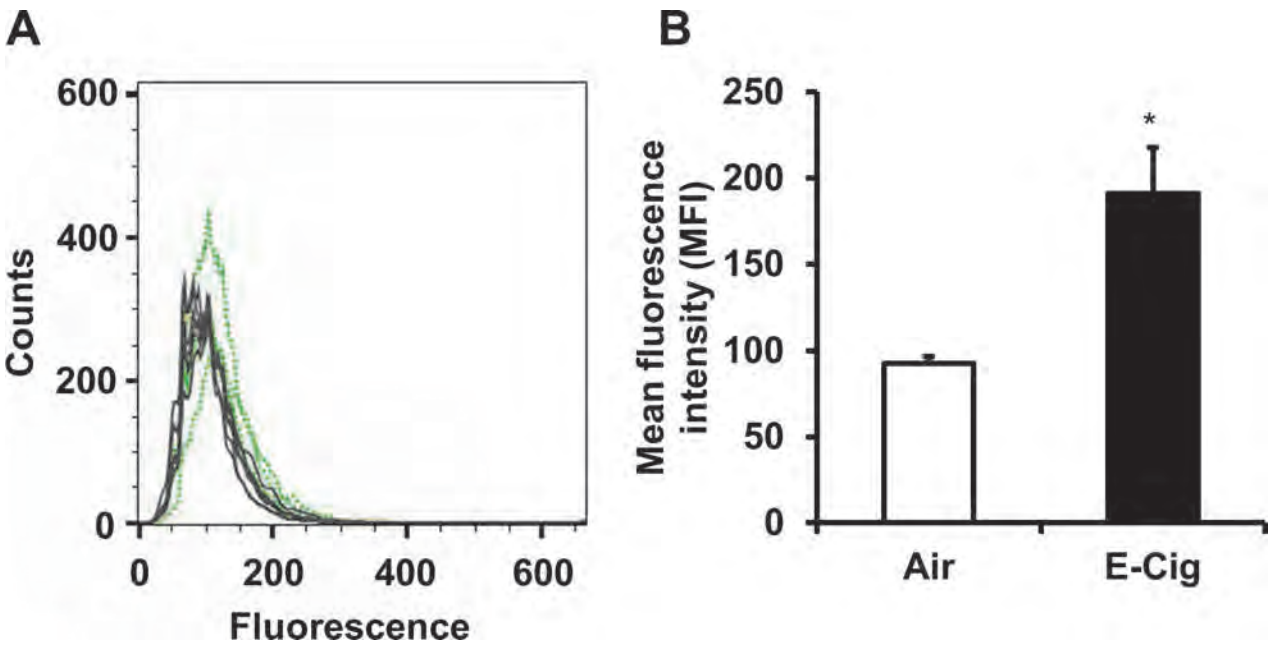

Fig 6. Air-liquid interface deposition of fluorescent substance on human bronchial airway epithelial cells. Beas-2B cells exposed to Blu e-cigarette vapor with a puff of 3–4 sec for 15 min. After exposure cells were immediately collected and measured by flow cytometry. (A) Histogram showing increase in non-specific fluorescence in cells exposed to e-cig aerosols. (B) Average fluorescence for e-cig exposed cells versus air-sham control shown as Mean Fluorescence Intensity (MFI). Data are shown as mean ± SD, n = 3, * $P < 0.05$ compared to air-sham control cells.

doi:10.1371/journal.pone.0116732.g006

E-cigarette aerosol exposure in mice caused lung inflammation and pro-inflammatory response

C57BL/6J mice were exposed to side-stream Classic tobacco flavor (16 mg nicotine) e-cig aerosols for 3 days (acute exposure). On average, macrophage counts were higher in e-cigs exposed mice, but were not statistically different compared to air group controls (Fig. 7A). The total cell counts in BAL fluid 24 hrs after the last exposure exhibited higher average numbers of cells, yet were not significant compared to air group controls (Fig. 7B). Analysis of BAL fluid collected 24 hrs after the last exposure (3rd day) to aerosols demonstrated pulmonary inflammation. MCP-1, a potent macrophage chemotactic cytokine was significantly increased in e-cigs aerosol exposed mice compared to air group controls (Fig. 7B). Levels of IL-6 which modulates a number of immune-inflammatory pathways in target leukocytes is significantly increased in BAL fluid from e-cigs exposed mice compared to air group controls (Fig. 7B).

To further assess the inflammatory response to side-stream e-cigs aerosol in mouse lung, a panel of cytokines/chemokines in BALF was measured in room air and e-cigs aerosol exposed mice using a Luminex kit (see Materials and Methods). Levels of IL-1α and IL-13 were significantly increased in e-cigs aerosol exposed mice compared to air group controls (Fig. 7C). Levels of IL-17, GM-CSF, IP-10, and MIP-2 in BALF did not change in response to e-cig side-stream aerosol. Levels of IL-1α, IL-1β, and IL-13 were slightly increased in e-cig aerosol exposed mice but not significant compared to air group controls. These data indicate that acute side-stream exposure to e-cig aerosol in mouse lung is sufficient to elicit an inflammatory response due to increased levels of proinflammatory mediators.

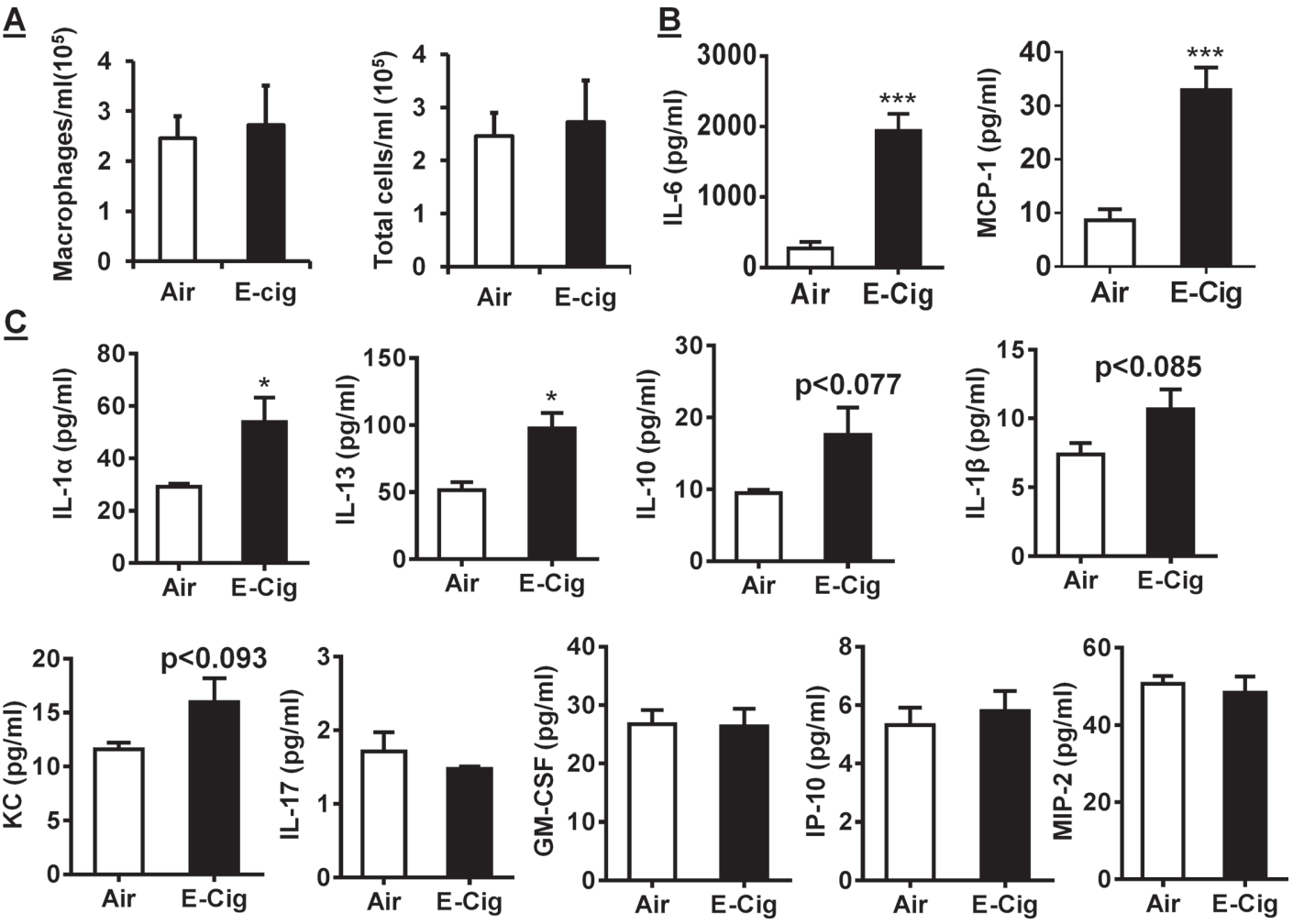

Fig 7. Acute e-cigarette aerosol exposure causes lung inflammation and pro-inflammatory response in mouse lungs. WT Mice (C57BL/6J) were exposed to e-cigarette aerosol exposure (200 mg/m^3 TPM) for 3 days and sacrificed 24 hrs after the last exposure. (A) At least 500 cells in the bronchoalveolar lavage fluid (BALF) were counted with hemocytometer to determine the number of macrophages and total cells on cytospin slides stained with Diff-Quik. (B) Levels of pro-inflammatory mediators MCP-1 and IL-6 were measured in BAL fluid obtained from room air and e-cig aerosol exposed mice (C57BL/6J). Data are shown as mean ± SD. *** $P < 0.001$ compared to air group mice (C) Cytokine/chemokine levels in BAL fluid from room air and e-cig aerosol exposed mice were also measure using Luminex multiplex assay. Data are shown as mean ± SEM, n = 3, *$P < 0.05$ compared to air group mice (C57BL/6J).

doi:10.1371/journal.pone.0116732.g007

Plasma cotinine levels in e-cig vapor exposed mice

Blood collected from mice sacrificed immediately after acute Blu e-cig exposure (3rd day after 5 hrs exposure) was used to measure cotinine levels, a nicotine metabolite [39]. Plasma cotinine levels reached an average of 10.78 ± 7.80 ng/ml for e-cig exposed mice. The plasma from mice sacrificed 24 hrs after the last exposure did not show any detectable cotinine levels (ND) after e-cigs exposure.

Intracellular glutathione levels in mouse lung exposed to short-term chronic e-cigarette aerosols

Both total and oxidized forms of glutathione were assessed. Glutathione levels in mouse lung lysates following animal exposure to side-stream Blu e-cig aerosols were depleted.

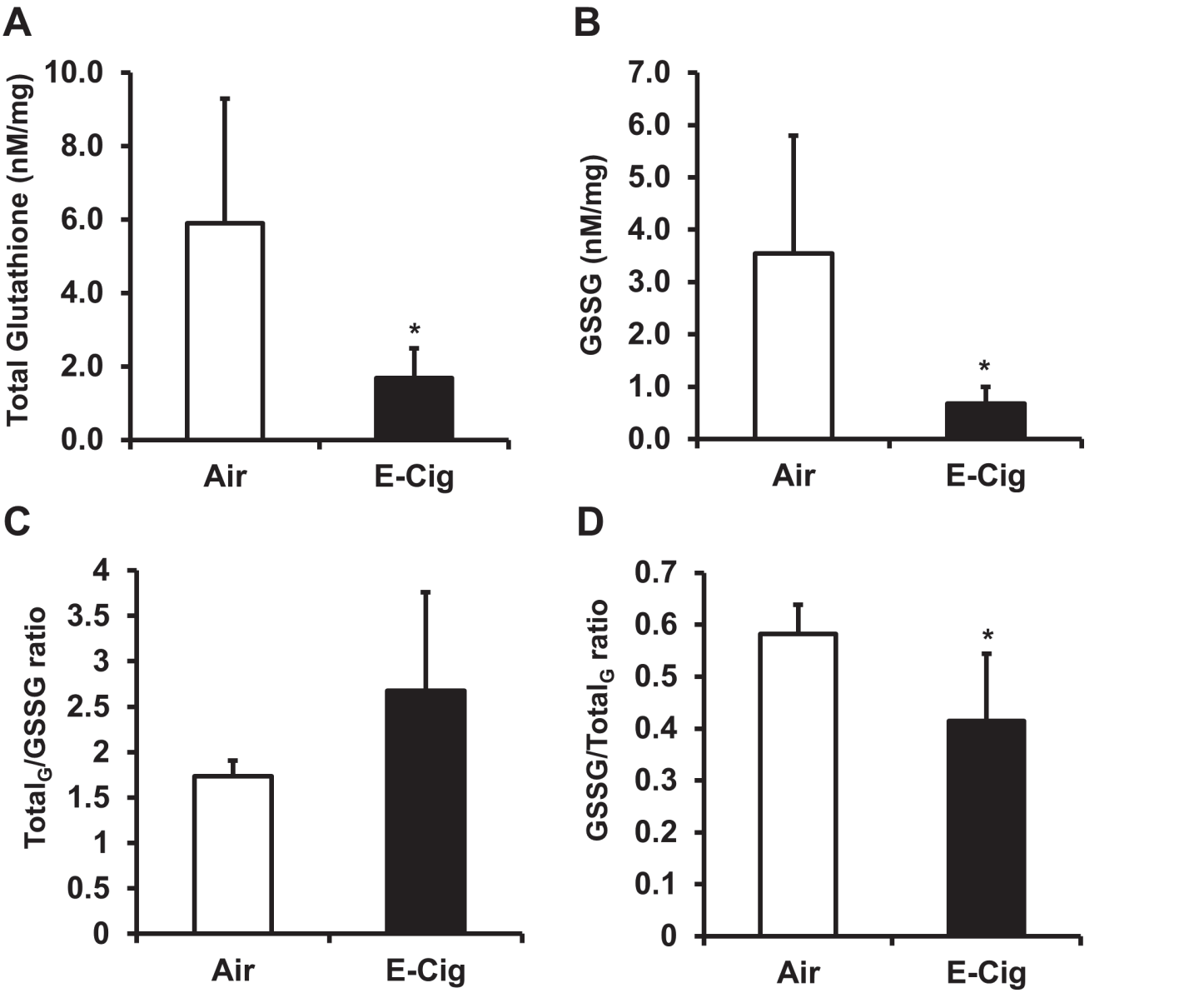

Fig 8. Intracellular glutathione levels in mouse lung following acute e-cigarette aerosol exposure. Mice were exposed to e-cig aerosol exposure (200 mg/m^3 TPM) for 3 days and sacrificed immediately after the last exposure (3rd day after 5 hrs exposure). Levels of (A) Total glutathione. (B) glutathione disulfide GSSG. (C) Total glutathione to GSSG ratio and (D) GSSG to total glutathione ratio were measured in lung homogenates. Data are shown as mean ± SD (n = 3/group).* $P < 0.05$ compared to air group mice (C57BL/6J).

doi:10.1371/journal.pone.0116732.g008

Air group levels of glutathione averaged 5.89 ± 3.40 nM/mg protein while the average glutathione level for animals exposed to e-cig aerosols was reduced to 1.69 ± 8.10 nM/mg protein (Fig. 8A). Oxidized (glutathione disulfide) levels of glutathione (GSSG) for air group averaged 3.54 ± 2.25 nM/mg protein and were decreased to 0.68 ± 0.32 nM/mg protein in exposed animals (Fig. 8B).

Ratios of total Glutathione to GSSG and *vice versa* were measured to determine if there was an effect from the side-stream e-cig aerosols on the balance between reduced and oxidized forms of glutathione within the lung. The ratio for total glutathione to GSSG was not significantly different between Air group and e-cig exposed animals (Fig. 8C). There was however, a

small decrease in the ratio for GSSG to total glutathione (**Fig. 8D**). These results suggests that total glutathione levels are reduced by e-cig aerosols and the redox balance between the reduced and oxidized forms of glutathione is affected by side-stream e-cig aerosol inhalation as well.

Discussion

ENDS/e-cigs have become prominent fixture in the consumer landscape. Habitually inhaling their aerosols has been implicated by manufacturers as a safer alternative to smoking conventional cigarettes and many electronic cigarette users have adopted similar perspectives [40]. However, recent e-cigs studies showing that there are substantial levels of nanoscale particles in addition to detectable levels of metals with toxic materials (e.g., aluminum, copper, magnesium, zinc, lead, chromium, manganese, and nickel) in e-cig aerosols brings this view into question [10]. At the nanoscale size, particles may reach the alveolar epithelium and mediate oxidative stress and inflammation [41,42].

It is not yet certain what the exact factors are associated with ENDS that might mediate oxidative stress. The eGO Vision ENDS vaporizer with refillable chamber and exchangeable heating element were employed to detect reactive OX/ROS under a variable set of parameters, such as the ratios of pure humectant mixtures, commercially available e-liquids, changeable voltage settings, and state of heating element. The ability to manipulate these parameters individually facilitates determination of the OX/ROS source from the vaporizer.

Using the above parameters, our results indicate that there are a number of variables that affect OX/ROS production in ENDS/e-cigs that are not exclusive to e-liquid aerosols. For example, it was observed that OX/ROS reactivity in aerosols produced from the refillable ENDS device varied between relatively high or low levels. For instance, in some cases DCF fluorescence values from refillable ENDS aerosols approached or overlapped air-sham control values despite aerosols being sufficiently produced during experimental trials that included vaporization of e-liquids/humectants. Multiple batches of DCFH solution prepared for additional experimental replicates may account for some of the variability seen for OX/ROS reactivity. The attainment of a number of unusually high fluorescence DCF measurements prompted us to question if the state of the heating element also influenced OX/ROS release from the device. We also noticed that each time a new heating element was installed into the eGo ENDS, a small amount of aerosol could be produced without addition of any e-liquid suggesting there may be volatile substances associated with ENDS heating elements following manufacturing.

The trend called "dripping" is intended to allow the user to achieve stronger 'hits' and also gives the option to more easily switch between flavors, brands, or nicotine content without frequently emptying and refilling the clearomizer chamber (communication with Mr. Douglas Done University of Rochester Department of Public Health Sciences) [43,44]. The design of refillable ENDS is suggested to incorporate "dripping" as an option for consumers [45]. The spike in OX/ROS release that resulted in high range DCF fluorescence after dripping e-liquid onto the 4th use heating element wick led us to hypothesize that "dripping" rather than filling the clearomizer with e-liquid, which completely submerges the heating element, is potentially more hazardous. Our results indicate that the dripping method for ENDS usage is likely to generate a larger amount of OX/ROS.

As Goniewicz *et al* reports, heating e-liquids with sufficient temperatures produces detectible levels of formaldehyde, acrolein, and acetaldehyde carbonyls with the possibility that these compounds form due to the pyrolysis of glycerin [5,6]. However, in comparison to conventional cigarette smoke, levels of these carbonyls were found to be between 9 and 807 times lower suggesting that ENDS/e-cigs may be a safer alternative to conventional cigarettes [5,6].

Carbonyl levels in ENDS/e-cigs also appeared to depend on the brand of the device while the black deposits that we see from the heating elements following our experiments are consistent with what has been found associated with other devices [10,46]. Therefore, although toxic carbonyl by-products measured in ENDS aerosols may be orders of magnitude lower than conventional cigarettes as reported by Goniewicz *et al.* and Kosmider *et al.*, the potential for delivering oxidizing agents as measured here may be currently underappreciated. The OX/ROS produced by "dripping" techniques that we observe coincides with emerging "vaping" trends that may place consumers at greater risk for lung damage. The higher volume of liquid surrounding the heating element when liquid is filled into the clearomizer during vaporization may implement an important cooling effect that prevents the device from producing temperatures high enough to form higher levels of combustion products or might mitigate the amount of OX/ROS released.

The nicotine containing e-liquids exhibited less reactivity with DCFH potentially due to low OX/ROS properties of nicotine in an aqueous solution [47]. Although comparison of the 0 mg and 24 mg nicotine Vape Dudes e-liquid aerosols produced by the refillable ENDS did not appear to be statistically different in DCF fluorescence to one another (large sampling error), the aerosols produced from the nicotine containing e-liquid was on average less than the samples without nicotine. This supports a possible trend in the reduction of OX/ROS in the presence of nicotine because the aerosols produced from the Blu e-cig cartomizer containing 16 mg nicotine exhibits a significant reduction in OX/ROS compared to the nicotine free cartomizer. Similarly, we observed unvaporized nicotine containing e-liquid was less oxidative to DCFH. Therefore, nicotine vaporized using either device, at least does not appear to contribute to OX/ROS generated.

E-liquid added directly to lung cells affects cell morphology, induces a stress phenotype and contributes to inflammatory response in a manner dependent on nicotine content and flavor choice. In this study, cinnamon flavored e-liquid elicited a strong IL-8 response compared to CSE which is consistent with the ability of cinnamon flavored e-liquids to induce cellular toxicity [2,3,48]. When cells are treated with nicotine containing e-liquid, filopodia appear to shorten similar to loss of filopodia in periodontal ligament gingiva fibroblasts treated with nicotine [49]. The accumulation of vacuoles we observe in cells treated with nicotine e-liquid is corroborated as well in other cells [37,49]. Pure propylene glycol and glycerin did not appreciably react with DCFH. However, cell morphology is affected by these humectants when cells were treated with them at the concentrations used in this study. The tobacco flavoring and possibly other additives exacerbated cell stress (enlargement, appearance of vacuoles). Propylene glycol and glycerin used in are FDA approved for use in foods, cosmetics, tobacco products, and is Generally Recognized as Safe (GRAS) [50]. However, direct exposure of these humectants to lung tissue and the concentrations that may accumulate in the lung from chronic ENDS use is not known. Propylene glycol may enhance the delivery of e-liquid additives of nicotine, flavors, and potential toxic impurities into the lung tissue. As skin penetration enhancer, propylene glycol is a mode of choice for transdermal drug delivery [51].

A limited number of studies have assessed the effect of commercially available e-liquids on cell toxicity and viability and attribute most of the toxicity being due to flavor additives [2]. The lung fibroblasts we treated with e-liquids showed no significant decrease in cell viability unless they were cultured in small wells with fewer numbers/density of cells. Mouse 3T3 fibroblast and rat myocardial cells treated with e-cig extracts were also minimally toxic and did not show appreciable effect on viability [3,52]. Other studies show cultured lung alveolar cells exposed to electronic cigarette aerosols rather than extract suggests that e-cigs induce toxicity in lung epithelial cells and lead to reduced viability in a manner that is dependent on flavor additive [53].

Epithelial airway H292 cells exposed to e-cig aerosols by air-liquid interface secrete proinflammatory cytokines, such as IL-6 and IL-8 into culture media after the cells were allowed to

culture for a 16 hour response. Although, lower levels of particulate matter have been measured in e-cig aerosols compared to conventional cigarette smoke [54], it is not well understood how these particles affect inflammation in the lung. How ENDS/e-cigs compare to conventional cigarettes in mediating inflammatory responses will require further experiments in various settings, conditions, and cell lines to under the mechanisms.

Mouse exposure to e-cig aerosols was carried out with a modified a Teague smoke exposure machine. Generally this machine is employed for standardized small animal exposures to side-stream/second-hand smoke of conventional cigarettes [32,33]. BALF from wild type mice (C57BL/6J) at 24 hrs following exposure to short-term chronic Blu e-cig aerosols (3 days) showed inflammatory response as indicated by increased inflammatory mediators. Though we did not observe an appreciable difference in macrophage lung influx or altered levels of total cells in BALF from mice exposed to e-cig aerosols, cytokine MCP-1 which acts a macrophage chemokine was significantly elevated in mouse BALF. IL-6, which is a potent mediator of acute-phase inflammatory response, was also significantly elevated in BALF from mice exposed to e-cig aerosols. The small elevation in average macrophage and total cell levels in BALF after aerosol exposures, though not significantly different than ambient air-group, is in line with the increased levels of various cytokines we measured that have modulatory roles in immunity and inflammation.

The cotinine levels in e-cig exposed WT (C57BL/6J) mice fall within the same range as C57BL/6J mice exposed to side-stream cigarette smoke for 6 hours in addition to passive smoking by human non-smokers cohabitating with smokers [55,56]. Therefore, nicotine is indeed delivered into mouse blood using the Teague smoke exposure machine which is designed for passive second hand smoke. Inhalation of nicotine is sufficient to increase cotinine levels in the blood which has been associated with tobacco smoke induced emphysema in mice [57]. This highlights that ENDS may be harmful and injurious by chronic consumption.

Mouse exposure to conventional cigarette smoke for acute exposure has been shown to sufficiently diminish glutathione levels in the lung in a strain-dependent fashion [58]. Exposure of C57BL/6J mice to acute exposure of e-cig aerosols also decreased total and oxidized levels of lung glutathione. Altering the glutathione levels in lung cells through inhalation of e-cig aerosols could impose oxidative stress culminating in inflammatory response as seen by conventional cigarette smoke [1,25,26,38].

The Forum of International Respiratory Societies has recommended that ENDS/e-cigs sales be restricted until their safety is better evaluated due to the limited amount of information addressing health risks associated with ENDS/e-cigs use [59]. However, based on our data, the ENDS devices warrant regulation and not to be condoned as a method to transition away from conventional cigarette addiction. Nevertheless, further long-term/chronic studies are required to evaluate the health risks of ENDS/e-cigs.

In conclusion, we showed that 1) OX/ROS are generated by vaporizing ENDS/e-cig e-liquids/e-juices and are further influenced by the state of the heating element, 2) differences in OX/ROS reactivity in e-liquids prior to vaporization is associated with e-liquid flavor, 3) e-liquids can mediate effects on lung cell morphology and affect viability, 4) e-cig aerosols can modulate levels of oxidative stress and inflammation markers in both lung cells and mouse lungs, and 5) e-cig aerosols affect *in vivo* in lung glutathione redox physiology implicating oxidative stress. These data clearly demonstrate the lung toxicity and hazards of exposure to ENDS/e-cigarettes.

Acknowledgments

The authors thank Dr. Alison Elder and her lab members for their technical assistance in cell-free ROS assay.

Author Contributions

Conceived and designed the experiments: CAL IKS HY IR. Performed the experiments: CAL IKS JG. Analyzed the data: CAL IKS. Contributed reagents/materials/analysis tools: DJO SM RR. Wrote the paper: CAL IKS DJO IR.

References

1. Yao H, Rahman I (2011) Current concepts on oxidative/carbonyl stress, inflammation and epigenetics in pathogenesis of chronic obstructive pulmonary disease. Toxicol Appl Pharmacol 254: 72–85. doi: 10.1016/j.taap.2009.10.022 PMID: 21296096
2. Bahl V, Lin S, Xu N, Davis B, Wang YH, et al. (2012) Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models. Reprod Toxicol 34: 529–537. doi: 10.1016/j.reprotox.2012.08.001 PMID: 22989551
3. Farsalinos KE, Romagna G, Allifranchini E, Ripamonti E, Bocchietto E, et al. (2013) Comparison of the cytotoxic potential of cigarette smoke and electronic cigarette vapour extract on cultured myocardial cells. Int J Environ Res Public Health 10: 5146–5162. doi: 10.3390/ijerph10105146 PMID: 24135821
4. Cheng T (2014) Chemical evaluation of electronic cigarettes. Tob Control 23 Suppl 2: ii11–17. doi: 10.1136/tobaccocontrol-2013-051482 PMID: 24732157
5. Kosmider L, Sobczak A, Fik M, Knysak J, Zaciera M, et al. (2014) Carbonyl Compounds in Electronic Cigarette Vapors-Effects of Nicotine Solvent and Battery Output Voltage. Nicotine Tob Res. 16: 1319–1326. doi: 10.1093/ntr/ntu078 PMID: 24832759
6. Goniewicz ML, Knysak J, Gawron M, Kosmider L, Sobczak A, et al. (2014) Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. Tob Control 23: 133–139. doi: 10.1136/tobaccocontrol-2012-050859 PMID: 23467656
7. Schripp T, Markewitz D, Uhde E, Salthammer T (2013) Does e-cigarette consumption cause passive vaping? Indoor Air 23: 25–31. doi: 10.1111/j.1600-0668.2012.00792.x PMID: 22672560
8. Paschke A, Scherer G, Heller WD (2002) Effects of ingredients on cigarette smoke composition and biological activity: A literature overview. Tobacco Research 20.
9. Fuoco FC, Buonanno G, Stabile L, Vigo P (2014) Influential parameters on particle concentration and size distribution in the mainstream of e-cigarettes. Environ Pollut 184: 523–529. doi: 10.1016/j.envpol.2013.10.010 PMID: 24172659
10. Williams M, Villarreal A, Bozhilov K, Lin S, Talbot P (2013) Metal and silicate particles including nanoparticles are present in electronic cigarette cartomizer fluid and aerosol. PLoS One 8: e57987. doi: 10.1371/journal.pone.0057987 PMID: 23526962
11. Nelson N (2014) More questions than answers surrounding e-cigarette debate. J Natl Cancer Inst 106: dju101. doi: 10.1093/jnci/dju101 PMID: 24685930
12. Schober W, Szendrei K, Matzen W, Osiander-Fuchs H, Heitmann D, et al. (2014) Use of electronic cigarettes (e-cigarettes) impairs indoor air quality and increases FeNO levels of e-cigarette consumers. Int J Hyg Environ Health 217: 628–637. doi: 10.1016/j.ijheh.2013.11.003 PMID: 24373737
13. Lim HH, Shin HS (2013) Measurement of Aldehydes in Replacement Liquids of Electronic Cigarettes by Headspace Gas Chromatography-mass Spectrometry. Bulletin of the Korean Chemical Society 34: 2691–2696.
14. Burstyn I (2014) Peering through the mist: systematic review of what the chemistry of contaminants in electronic cigarettes tells us about health risks. BMC Public Health 14: 18. doi: 10.1186/1471-2458-14-18 PMID: 24406205
15. Sundar IK, Yao H, Rahman I (2013) Oxidative stress and chromatin remodeling in chronic obstructive pulmonary disease and smoking-related diseases. Antioxid Redox Signal 18: 1956–1971. doi: 10.1089/ars.2012.4863 PMID: 22978694
16. Pryor WA, Stone K (1993) Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. Ann N Y Acad Sci 686: 12–27; discussion 27–18. PMID: 8512242
17. Jomova K, Valko M (2011) Advances in metal-induced oxidative stress and human disease. Toxicology 283: 65–87. doi: 10.1016/j.tox.2011.03.001 PMID: 21414382
18. Black MJ, Brandt RB (1974) Spectrofluorometric analysis of hydrogen peroxide. Anal Biochem 58: 246–254. PMID: 4825377
19. Myhre O, Andersen JM, Aarnes H, Fonnum F (2003) Evaluation of the probes 2',7'-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. Biochem Pharmacol 65: 1575–1582. PMID: 12754093

20. Sitzwohl C, Langheinrich A, Schober A, Krafft P, Sessler DI, et al. (2010) Endobronchial intubation detected by insertion depth of endotracheal tube, bilateral auscultation, or observation of chest movements: randomised trial. BMJ 341: c5943. doi: 10.1136/bmj.c5943 PMID: 21062875

21. Hung HF, Wang CS (2001) Experimental determination of reactive oxygen species in Taipei aerosols. Journal of Aerosol Science 32: 1201–1211.

22. Jiang J, Oberdorster G, Elder A, Gelein R, Mercer P, et al. (2008) Does Nanoparticle Activity Depend upon Size and Crystal Phase? Nanotoxicology 2: 33–42. PMID: 20827377

23. Hua M, Yip H, Talbot P (2013) Mining data on usage of electronic nicotine delivery systems (ENDS) from YouTube videos. Tob Control 22: 103–106. doi: 10.1136/tobaccocontrol-2011-050226 PMID: 22116832

24. Huang MF, Lin WL, Ma YC (2005) A study of reactive oxygen species in mainstream of cigarette. Indoor Air 15: 135–140. PMID: 15737156

25. Kode A, Yang SR, Rahman I (2006) Differential effects of cigarette smoke on oxidative stress and proinflammatory cytokine release in primary human airway epithelial cells and in a variety of transformed alveolar epithelial cells. Respir Res 7: 132. PMID: 17062156

26. Moodie FM, Marwick JA, Anderson CS, Szulakowski P, Biswas SK, et al. (2004) Oxidative stress and cigarette smoke alter chromatin remodeling but differentially regulate NF-kappaB activation and proinflammatory cytokine release in alveolar epithelial cells. FASEB J 18: 1897–1899. PMID: 15456740

27. Yang SR, Chida AS, Bauter MR, Shafiq N, Seweryniak K, et al. (2006) Cigarette smoke induces proinflammatory cytokine release by activation of NF-kappaB and posttranslational modifications of histone deacetylase in macrophages. Am J Physiol Lung Cell Mol Physiol 291: L46–57. PMID: 16473865

28. Phillips J, Kluss B, Richter A, Massey E (2005) Exposure of bronchial epithelial cells to whole cigarette smoke: assessment of cellular responses. Altern Lab Anim 33: 239–248. PMID: 16180978

29. Maunders H, Patwardhan S, Phillips J, Clack A, Richter A (2007) Human bronchial epithelial cell transcriptome: gene expression changes following acute exposure to whole cigarette smoke in vitro. Am J Physiol Lung Cell Mol Physiol 292: L1248–1256. PMID: 17220372

30. Thorne D, Wilson J, Kumaravel TS, Massey ED, McEwan M (2009) Measurement of oxidative DNA damage induced by mainstream cigarette smoke in cultured NCI-H292 human pulmonary carcinoma cells. Mutat Res 673: 3–8. doi: 10.1016/j.mrgentox.2008.11.008 PMID: 19100859

31. Adamson J, Azzopardi D, Errington G, Dickens C, McAughey J, et al. (2011) Assessment of an in vitro whole cigarette smoke exposure system: The Borgwaldt RM20S 8-syringe smoking machine. Chem Cent J 5: 50. doi: 10.1186/1752-153X-5-50 PMID: 21867559

32. Hwang JW, Sundar IK, Yao H, Sellix MT, Rahman I (2014) Circadian clock function is disrupted by environmental tobacco/cigarette smoke, leading to lung inflammation and injury via a SIRT1-BMAL1 pathway. FASEB J 28: 176–194. doi: 10.1096/fj.13-232629 PMID: 24025728

33. Yao H, Chung S, Hwang JW, Rajendrasozhan S, Sundar IK, et al. (2012) SIRT1 protects against emphysema via FOXO3-mediated reduction of premature senescence in mice. J Clin Invest 122: 2032–2045. doi: 10.1172/JCI60132 PMID: 22546858

34. Rahman I, Kode A, Biswas SK (2006) Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. Nat Protoc 1: 3159–3165. PMID: 17406579

35. Winterbourn CC (2014) The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells. Biochim Biophys Acta 1840: 730–738. doi: 10.1016/j.bbagen.2013.05.004 PMID: 23665586

36. Goniewicz ML, Kuma T, Gawron M, Knysak J, Kosmider L (2013) Nicotine levels in electronic cigarettes. Nicotine Tob Res 15: 158–166. doi: 10.1093/ntr/nts103 PMID: 22529223

37. Laytragoon-Lewin N, Bahram F, Rutqvist LE, Turesson I, Lewin F (2011) Direct effects of pure nicotine, cigarette smoke extract, Swedish-type smokeless tobacco (Snus) extract and ethanol on human normal endothelial cells and fibroblasts. Anticancer Res 31: 1527–1534. PMID: 21617206

38. Baglole CJ, Bushinsky SM, Garcia TM, Kode A, Rahman I, et al. (2006) Differential induction of apoptosis by cigarette smoke extract in primary human lung fibroblast strains: implications for emphysema. Am J Physiol Lung Cell Mol Physiol 291: L19–29. PMID: 16443644

39. Goniewicz ML, Eisner MD, Lazcano-Ponce E, Zielinska-Danch W, Koszowski B, et al. (2011) Comparison of urine cotinine and the tobacco-specific nitrosamine metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and their ratio to discriminate active from passive smoking. Nicotine Tob Res 13: 202–208. doi: 10.1093/ntr/ntq237 PMID: 21330276

40. Goniewicz ML, Lingas EO, Hajek P (2013) Patterns of electronic cigarette use and user beliefs about their safety and benefits: an internet survey. Drug Alcohol Rev 32: 133–140. doi: 10.1111/j.1465-3362.2012.00512.x PMID: 22994631

41. Manke A, Wang L, Rojanasakul Y (2013) Mechanisms of nanoparticle-induced oxidative stress and toxicity. Biomed Res Int 2013: 942916. doi: 10.1155/2013/942916 PMID: 24027766

42. Bakand S, Hayes A, Dechsakulthorn F (2012) Nanoparticles: a review of particle toxicology following inhalation exposure. Inhal Toxicol 24: 125–135. doi: 10.3109/08958378.2010.642021 PMID: 22260506

43. Weaver M, Breland A, Spindle T, Eissenberg T (2014) Electronic cigarettes: a review of safety and clinical issues. J Addict Med 8: 234–240. doi: 10.1097/ADM.0000000000000043 PMID: 25089953

44. JK P (2014) Waterpipes and Electronic Cigarettes: Increasing Prevalence and Expanding Science. Chemical Research in Toxicology 27: 1336–1343. doi: 10.1021/tx500200j PMID: 25338174

45. Brown CJ, Cheng JM (2014) Electronic cigarettes: product characterisation and design considerations. Tob Control 23 Suppl 2: ii4–10. doi: 10.1136/tobaccocontrol-2013-051476 PMID: 24732162

46. Uchiyama S, Ohta K, Inaba Y, Kunugita N (2013) Determination of carbonyl compounds generated from the E-cigarette using coupled silica cartridges impregnated with hydroquinone and 2,4-dinitrophenylhydrazine, followed by high-performance liquid chromatography. Anal Sci 29: 1219–1222. PMID: 24334991

47. Kosno K, Celuch M, Mirkowski J, Janik I, Pogocki D (2012) FREE RADICAL REACTIONS OF NICOTINE. Prof Jacek Michalik, Ph D, D Sc Wiktor Smułek, Ph D: 20.

48. Behar RZ, Davis B, Wang Y, Bahl V, Lin S, et al. (2014) Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids. Toxicol In Vitro 28: 198–208. PMID: 24516877

49. Alpar B, Leyhausen G, Sapotnick A, Gunay H, Geurtsen W (1998) Nicotine-induced alterations in human primary periodontal ligament and gingiva fibroblast cultures. Clin Oral Investig 2: 40–46. PMID: 9667154

50. Arnold C (2014) Vaping and health: what do we know about e-cigarettes? Environ Health Perspect 122: A244–249. doi: 10.1289/ehp.122-A244 PMID: 25181730

51. Lane ME (2013) Skin penetration enhancers. Int J Pharm 447: 12–21. doi: 10.1016/j.ijpharm.2013.02.040 PMID: 23462366

52. Romagna G, Allifranchini E, Bocchietto E, Todeschi S, Esposito M, et al. (2013) Cytotoxicity evaluation of electronic cigarette vapor extract on cultured mammalian fibroblasts (ClearStream-LIFE): comparison with tobacco cigarette smoke extract. Inhal Toxicol 25: 354–361. doi: 10.3109/08958378.2013.793439 PMID: 23742112

53. Cervellati F, Muresan XM, Sticozzi C, Gambari R, Montagner G, et al. (2014) Comparative effects between electronic and cigarette smoke in human keratinocytes and epithelial lung cells. Toxicol In Vitro 28: 999–1005. doi: 10.1016/j.tiv.2014.04.012 PMID: 24809892

54. Ruprecht AA, De Marco C, Pozzi P, Munarini E, Mazza R, et al. (2014) Comparison between particulate matter and ultrafine particle emission by electronic and normal cigarettes in real-life conditions. Tumori 100: e24–27. doi: 10.1700/1430.15833 PMID: 24675506

55. Jarvis MJ, Feyerabend C, Bryant A, Hedges B, Primatesta P (2001) Passive smoking in the home: plasma cotinine concentrations in non-smokers with smoking partners. Tob Control 10: 368–374. PMID: 11740030

56. Horn KH, Esposito ER, Greene RM, Pisano MM (2008) The effect of cigarette smoke exposure on developing folate binding protein-2 null mice. Reprod Toxicol 26: 203–209. doi: 10.1016/j.reprotox.2008.09.013 PMID: 18992323

57. Xu X, Su Y, Fan ZH (2014) Cotinine concentration in serum correlates with tobacco smoke-induced emphysema in mice. Sci Rep 4: 3864. doi: 10.1038/srep03864 PMID: 24463700

58. Yao H, Edirisinghe I, Rajendrasozhan S, Yang SR, Caito S, et al. (2008) Cigarette smoke-mediated inflammatory and oxidative responses are strain-dependent in mice. Am J Physiol Lung Cell Mol Physiol 294: L1174–1186. doi: 10.1152/ajplung.00439.2007 PMID: 18375740

59. Schraufnagel DE, Blasi F, Drummond MB, Lam DC, Latif E, et al. (2014) Electronic Cigarettes: A Position Statement of the Forum of International Respiratory Societies. Am J Respir Crit Care Med. 190: 611–618. doi: 10.1164/rccm.201407-1198PP PMID: 25006874

Leslie *BMC Medicine* 2013, **11**:27
http://www.biomedcentral.com/1741-7015/11/27

BMC Medicine

COMMENTARY Open Access

Multigenerational epigenetic effects of nicotine on lung function

Frances M Leslie

Abstract

A recent preclinical study has shown that not only maternal smoking but also grandmaternal smoking is associated with elevated pediatric asthma risk. Using a well-established rat model of *in utero* nicotine exposure, Rehan *et al*. have now demonstrated multigenerational effects of nicotine that could explain this 'grandmother effect'. F1 offspring of nicotine-treated pregnant rats exhibited asthma-like changes to lung function and associated epigenetic changes to DNA and histones in both lungs and gonads. These alterations were blocked by co-administration of the peroxisome proliferator-activated receptor-γ agonist, rosiglitazone, implicating downregulation of this receptor in the nicotine effects. F2 offspring of F1 mated animals exhibited similar changes in lung function to that of their parents, even though they had never been exposed to nicotine. Thus epigenetic mechanisms appear to underlie the multigenerational transmission of a nicotine-induced asthma-like phenotype. These findings emphasize the need for more effective smoking cessation strategies during pregnancy, and cast further doubt on the safety of using nicotine replacement therapy to reduce tobacco use in pregnant women.

Please see related article: http://www.biomedcentral.com/1741-7015/10/129

Keywords: development, DNA methylation, histone acetylation, nicotine replacement therapy (NRT), peroxisome proliferator-activated receptor-γ (PPARγ), smoking, tobacco

Background

The negative health effects of tobacco use in adult smokers are well established [1]. On average, smoking leads to more than 400,000 premature deaths in the United States each year, with an overall decrease in life expectancy of 14 years. The major adverse health consequences of smoking include cancer, cardiovascular disease and respiratory disorders. Since many women continue to smoke during pregnancy, the negative impact of tobacco can begin before birth [2]. Maternal smoking is now the single most important preventable risk factor for Sudden Infant Death Syndrome, which results from developmental delays in the neural control of cardiopulmonary function [1,2]. Children of smokers are also more prone to respiratory diseases, such as asthma. One surprising finding is that a grandmother's tobacco use is associated with increased risk of early childhood asthma, even if the mother did not smoke while pregnant [3]. Rehan *et al.* [4] have recently used a well-established rat model of *in utero* nicotine exposure to determine the possible mechanisms underlying this clinical observation (Figure 1). They found that maternal nicotine exposure exerted adverse effects on lung development, not only for the immediate offspring but also for the next generation. They also identified epigenetic mechanisms involved in this multigenerational transmission. This paper will review these groundbreaking findings and discuss their potential clinical implications.

Transgenerational transmission of nicotine effects

Within recent years, fundamental assumptions about genetic inheritance have been revisited [5,6]. In addition to classical Mendelian genetics, the environment has been shown to contribute to inherited characteristics by placing epigenetic tags on DNA or associated histones that result in modified gene expression. In particular, the prenatal environment can result in reprogramming of the epigenome, as demonstrated by Rehan *et al.* [4]. They showed that acute daily injections of nicotine throughout pregnancy led to epigenetic modifications of

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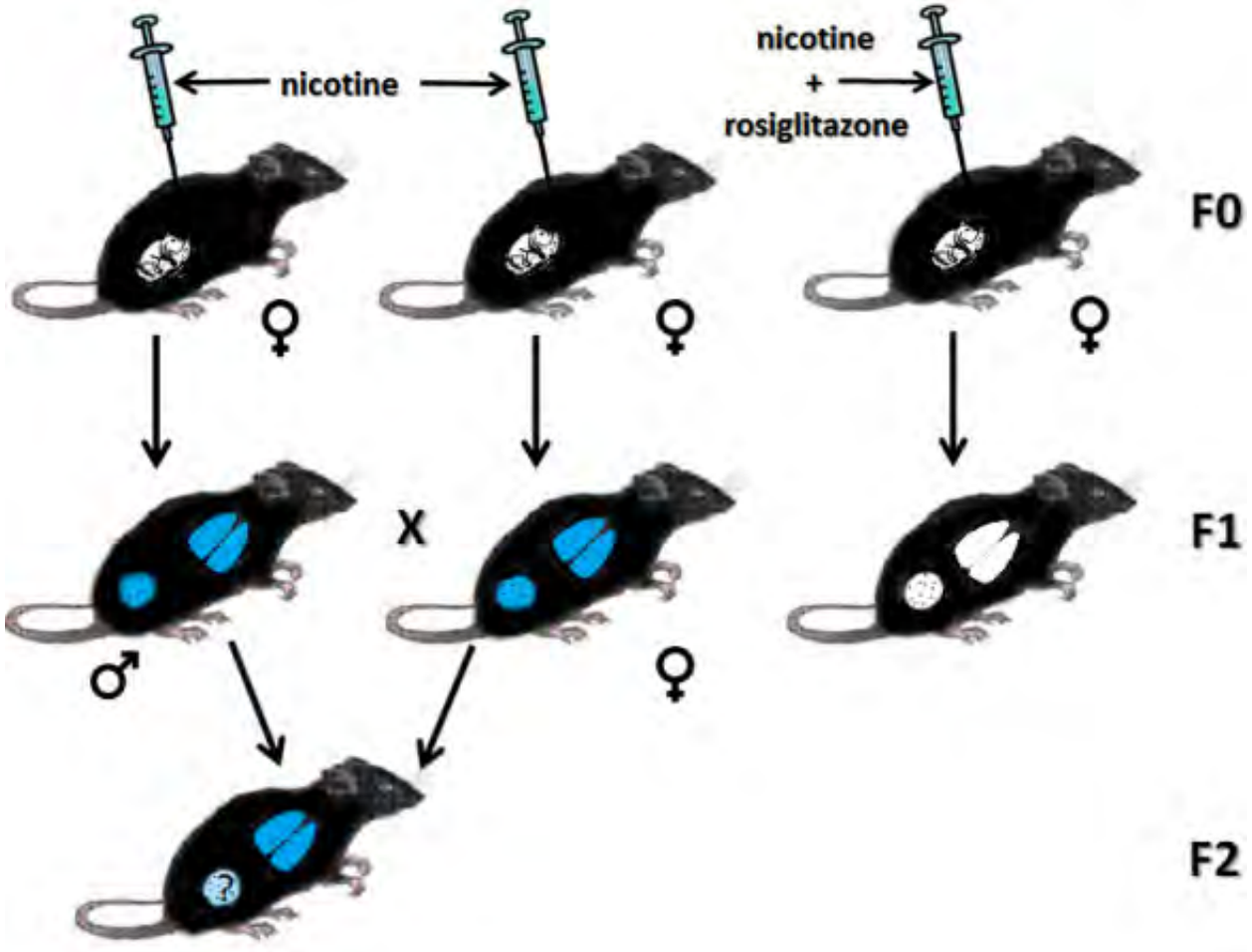

Figure 1 Multigenerational transmission of nicotine-induced effects. The diagram illustrates the experimental design and findings of Rehan *et al.* [4]. Pregnant dams (F0 generation) are injected with nicotine or nicotine + rosiglitazone. The lungs and gonads of both male and female offspring (F1 generation) of nicotine-treated dams exhibit epigenetic changes, and the lungs show an asthma-like functional phenotype (blue nicotine-induced changes). These nicotine effects are not seen in the offspring of animals treated with nicotine + rosiglitazone. Offspring of F1 mated pairs (F2 generation) exhibit the same nicotine-induced changes to lung function as their parents, even though they were not exposed to drug. It is not yet known whether the F2 offspring continue to exhibit alterations to the germ cell epigenome.

lung tissue in the F1 offspring (Figure 1), with a resulting asthma-like phenotype. When F1 rats were mated, similar changes in lung function were observed in their F2 offspring, even though they had never been exposed to nicotine. Nicotine induction of the asthma phenotype was found to result from a downregulation of mesenchymal peroxisome proliferator-activated receptor-γ (PPARγ), which plays a critical role in the development, homeostasis and repair of the lung [7]. Rosiglitazone, a PPARγ agonist, completely prevented the alterations in lung function, and in H3 acetylation of lung histones, when co-administered with nicotine to the pregnant dam [4].

Although the F2 rats had never been exposed to nicotine, their primordial germ cells were potential targets while the F1 parents were *in utero*. The finding of nicotine-induced epigenetic changes in both ovarian and testicular tissues from F1 generation rats provides support for this as a possible mechanism for functional changes observed in F2 offspring. Whereas H4 histone acetylation was increased in the gonads of both sexes, DNA methylation was increased in testes but decreased in ovaries. All of these epigenetic changes were eliminated by rosiglitazone, implicating the downregulation of PPARγ as a more universal mechanism of nicotine-induced changes to the epigenome, impacting germ cells as well as lung tissue.

In utero nicotine exposure resulted in alterations to both the somatic and germ cell epigenome. However, the nicotine-induced germ cell epigenetic changes were only

examined in the gonads of F1 offspring. The true test of whether nicotine can induce permanent epigenetic changes to the germline, with resulting transgenerational genetic inheritance, will require studies that look at germ cells in the F2 offspring and lung function in subsequent F3 and F4 generations. This issue notwithstanding, however, this preclinical study is critically important in that it provides the first experimental evidence for multigenerational effects of *in utero* nicotine exposure. Furthermore, it provides a conceptual framework with which to understand the novel clinical observation that grandmothers' smoking patterns are as important as that of the mother in determining pediatric lung function [3].

Future directions

Given the significant health risks to the offspring, effective smoking cessation strategies during pregnancy are clearly needed. Whereas behavioral therapy does not involve drug exposure to the fetus, it is not always effective. This has led some practitioners to advocate the use of nicotine replacement therapy (NRT) as a smoking cessation aid for pregnant women since it may reduce the risk of low birth weight and preterm delivery [8]. However, the clinical community is divided on this recommendation because of continued concerns about efficacy and safety [9,10]. Preclinical investigators have long argued that nicotine is a developmental teratogen and should not be used as a treatment for pregnant smokers [2,11]. The current finding of multigenerational effects of *in utero* nicotine exposure will provide critical support for this view.

Another issue that requires further assessment is whether nicotine can induce more generalized epigenetic changes, not only as a result of *in utero* exposure. Primordial germ cells are also exposed to nicotine in non-pregnant female and male smokers, and may undergo epigenetic changes that are transmitted to future progeny. Support for this concept comes from a recent study that found cigarette smoke to induce specific differences in the spermatozoal microRNA content of human smokers as compared to non-smokers [12]. The microRNAs that were differentially impacted mediate pathways required for healthy sperm and normal embryo development, suggesting a possible adverse impact of smoking on these processes. Smoking may also produce somatic changes in the developing brain during its critical adolescent period. Nearly all tobacco use begins in childhood and adolescence, with almost 90% of adult smokers reporting that they started smoking by the age of 18 [13]. A growing clinical and preclinical literature suggests that adolescent nicotine exposure produces unique and long-term changes in neural structure, function and resulting behavior [11,14]. Similar PPARγ-mediated epigenetic mechanisms to those identified by Rehan and colleagues [4] may underlie these unique developmental effects of nicotine during adolescence.

Findings of widespread nicotine-induced changes to the epigenome should stimulate further, more aggressive, efforts to restrict youth access to tobacco products. They may also lead to possible therapeutic strategies to prevent or reverse the adverse impact of nicotine exposure on the developing brain and periphery.

Abbreviations

NRT: nicotine replacement therapy; PPARγ: peroxisome proliferator-activated receptor-γ

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Competing interests

The author declares that they have no competing interests.

Received: 30 November 2012 Accepted: 4 February 2013
Published: 4 February 2013

References

1. U.S. Department of Health and Human Services: **The Health Consequences of Smoking: A Report of the Surgeon General.** Atlanta (GA): U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health; 2004.
2. Dwyer JB, Broide RS, Leslie FM: **Nicotine and brain development.** *Birth Defects Res C Embryo Today* 2008, **84**:30-44.
3. Li YF, Langholz B, Salam MT, Gilliland FD: **Maternal and grandmaternal smoking patterns are associated with early childhood asthma.** *Chest* 2005, **127**:1232-1241.
4. Rehan VK, Liu J, Naeem E, Tian J, Sakurai R, Kwong K, Akbari O, Torday JS: **Perinatal nicotine exposure induces asthma in second generation offspring.** *BMC Med* 2012, **10**:129.
5. Gabory A, Attig L, Junien C: **Sexual dimorphism in environmental epigenetic programming.** *Mol Cell Endocrinol* 2009, **304**:8-18.
6. Bonduriansky R: **Rethinking heredity, again.** *Trends Ecol Evol* 2012, **27**:330-336.
7. Rehan VK, Torday JS: **PPARγ signaling mediates the evolution, development, homeostasis, and repair of the lung.** *PPAR Res* 2012, **2012**:289867.
8. Forinash AB, Pitlick JM, Clark K, Alstat V: **Nicotine replacement therapy effect on pregnancy outcomes.** *Ann Pharmacother* 2010, **44**:1817-1821.
9. Oncken CA, Kranzler HR: **What do we know about the role of pharmacotherapy for smoking cessation before or during pregnancy?** *Nicotine Tob Res* 2009, **11**:1265-1273.
10. Milidou I, Henriksen TB, Jensen MS, Olsen J, Søndergaard C: **Nicotine replacement therapy during pregnancy and infantile colic in the offspring.** *Pediatrics* 2012, **129**:e652-658.
11. Slotkin TA: **If nicotine is a developmental neurotoxicant in animal studies, dare we recommend nicotine replacement therapy in pregnant women and adolescents?** *Neurotoxicol Teratol* 2008, **30**:1-19.
12. Marczylo EL, Amoako AA, Konje JC, Gant TW, Marczylo TH: **Smoking induces differential miRNA expression in human spermatozoa: a potential transgenerational epigenetic concern?** *Epigenetics* 2012, **7**:432-439.
13. U.S. Department of Health and Human Services: *Preventing Tobacco Use Among Youth and Young Adults: A Report of the Surgeon General* Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health; 2012.

14. Dwyer JB, McQuown SC, Leslie FM: **The dynamic effects of nicotine on the developing brain.** *Pharmacol Ther* 2009, **122**:125-139.

Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1741-7015/11/27/prepub

doi:10.1186/1741-7015-11-27
Cite this article as: Leslie: Multigenerational epigenetic effects of nicotine on lung function. *BMC Medicine* 2013 **11**:27.

Progress in Biophysics and Molecular Biology 118 (2015) 21–33

Contents lists available at ScienceDirect

Progress in Biophysics and Molecular Biology

journal homepage: www.elsevier.com/locate/pbiomolbio

Review

Multigenerational and transgenerational inheritance of drug exposure: The effects of alcohol, opiates, cocaine, marijuana, and nicotine

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ARTICLE INFO

Article history:
Available online 1 April 2015

Keywords:
Multigenerational
Transgenerational
Epigenetic
Drug abuse

ABSTRACT

Familial inheritance of drug abuse is composed of both genetic and environmental factors. Additionally, epigenetic transgenerational inheritance may provide a means by which parental drug use can influence several generations of offspring. Recent evidence suggests that parental drug exposure produces behavioral, biochemical, and neuroanatomical changes in future generations. The focus of this review is to discuss these multigenerational and transgenerational phenotypes in the offspring of animals exposed to drugs of abuse. Specifically, changes found following the administration of alcohol, opioids, cocaine, marijuana, and nicotine will be discussed. In addition, epigenetic modifications to the genome following administration of these drugs will be detailed as well as their potential for transmission to the next generation.

Contents

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http://dx.doi.org/10.1016/j.pbiomolbio.2015.03.002
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1. Introduction

Drug abuse impacts critical brain regions resulting in reward-seeking and craving, drug dependence, withdrawal, and alterations in both anxiety and learning and memory (Koob and Volkow, 2009). Clinical findings document familial patterns of drug use and dependence (Nielsen et al., 2012). Genome-wide association studies (GWAS) have identified a number of genes, chromosomal regions, and allelic variants likely to contribute to drug addiction, however not all inherited information is present in DNA sequence. Recently chemical alterations in the genome not related to DNA sequence variations have been identified following drug exposure and these epigenetic modifications may contribute to drug abuse and dependence across familial generations (Hughes, 2014). Specifically, several phenotypic consequences of drug exposure are found across multiple generations of offspring, despite no previous exposure to drug and no allelic or chromosomal variation (Skinner, 2008). Thus, epigenetic modifications could provide a mechanism underlying the longevity of psychiatric conditions such as drug abuse both within, as well as across generations (Nielsen et al., 2012).

The focus of this review is to discuss phenotypic variation (behavior, neurochemical, and structural) in offspring of drug-exposed parents and grandparents in animal models. Inheritance of behavioral changes will be limited to outcomes that measure drug response and reward sensitivity in animals. In addition, epigenetic modifications of gene regulation in response to or mediating the effects of drugs of abuse will be discussed. Finally, the transmission of epigenetic changes through the germ line as a mechanism that may underlie transgenerational inheritance will be reviewed.

2. Epigenetics

In an attempt to rectify the vast differences in use of the term *epigenetics*, Adrian Bird proposed a definition that encompasses both the chemical mechanisms as well as the necessity for inheritance: epigenetics is "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (Bird, 2007). Therefore, these chromosomal modifications can be sudden or accumulate over time in response to exposure to a stimulus and is, nevertheless, inherited in the absence of the signal or event that initiated the change (Bird, 2007). In addition, chemical alterations to the genome, also referred to as the epigenome when including the DNA packaging, can ultimately change the functional expression of genes (Bird, 2002). Epigenetic inheritance occurs by persistence of the modifications through several generations of cell division or animals (Bird, 2007).

Cellular epigenetic modifications in response to the environment include chromatin remodeling and DNA methylation (Skinner, 2011; Daxinger and Whitelaw, 2012). Further explanation of these modifications can be found in Box 1. There is some evidence that expression of a class of small noncoding RNAs known as micro RNAs (miRNAs) can transmit phenotypes to the next generation (Rassoulzadegan et al., 2006). While miRNAs have been implicated in the inheritance of stress (Gapp et al., 2014; Rodgers

Box 1
Epigenetic Modifications.

Chromatin Remodeling and Histone Modifications

Histone complexes contain an amino (N) terminal tail that can undergo modifications; these modifications "condense" or "relax" the state of DNA wrapped around the histone. Modifications include acetylation, methylation, phosphorylation, ubiquitylation, and SUMOylation, however for the purposes of this review only acetylation and methylation will be discussed.

Acetylation
Acetylation of histone tail relaxes chromatin and allows for gene transcription. For example acetylation of histone 3 (AcH3) and acetylation of lysine 14 of histone 3 (aceH3K14) are both correlated to increased transcription of target genes.

Methylation
Histone methylation can cause gene activation or repression depending on the residue undergoing modification. Histone 3 lysine 4 trimethylation (H3K4me3) causes the activation of gene transcription while histone 3 lysine 9 dimethylation (H3K9me2) association with a gene is correlated to repression of that gene.

For a thorough review on epigenetic inheritance mechanisms and specifically theories on histone modification transmission through DNA replication see (Martin and Zhang, 2007).

DNA methylation

DNA methylation regulates transcription by acting directly on the genome. In the past research has suggested that the presence of methyl groups suppresses gene expression, however this is not always the case. DNA methyltransferases (DNMTs) catalyze the addition of a methyl group onto a cytosine nucleotide that is usually positioned next to a guanine nucleotide (CpG). Methyl groups are donated by S-adenosyl methionine (SAM); therefore, the addition of a methyl onto the cytosine converts the cytosine to 5-methylcytosine. Gene regulation via DNA methylation is achieved through CpGs in the regulatory regions (promoters, enhancers, insulator) of genes.

et al., 2013), there is no evidence to suggest miRNA mediated effects of drug exposure on offspring. It is quite complex to establish a mechanism of inheritance that could account for altered behaviors, biochemical responses, and cellular morphology in the offspring of F0 exposed parents. Therefore, within each drug class epigenetic changes of interest within the animal exposed will be discussed as well as any evidence of generational inheritance of chemical modifications to the genome.

3. Epigenetics across generations

Epigenetic transgenerational inheritance is defined as "germline-mediated inheritance of epigenetic information between generations in the absence of direct environmental influences that leads to phenotypic variation" (Skinner, 2011). In contrast, multigenerational phenotypes are those derived from direct exposure to the drug. Thus, if drug exposure occurs in F0 males or females prior to pregnancy, the germ cells, which go on to produce the F1 generation are also "exposed". Therefore, phenotypes found in F0 and F1 animals are multigenerational and only those present in F2 animals are considered transgenerational as these F2 animals are the first generation whose cells have not been exposed to drug (Fig. 1, top).

In contrast, if F0 mothers are exposed to drug during pregnancy, the somatic and germ cells of the F1 offspring receive direct exposure to the drug *in utero* (Fig. 1, bottom). Since the germ cells of the F1 offspring are exposed to drug and the F2 offspring originate from these exposed germ cells, then the F0 parents, F1 and F2 offspring are all exposed to the drug (Skinner, 2008). In this case, F0, F1, and F2 phenotypes are multigenerational and only phenotypes observed in the F3 generation and beyond are transgenerational (Fig. 1).

4. Gamete development and reprogramming

Germline reprogramming events may directly facilitate epigenetic inheritance across generations. Genome wide DNA methylation reprogramming occurs at two time points in early embryonic development. Both reprogramming events are well understood in mice. First, genome-wide DNA demethylation occurs post-fertilization in the zygote to erase gamete epigenome methylation in order to promote cellular totipotency in the developing embryo. However, at this time genomic imprints, methylation on imprinted genes such as *H19* and *Igf2*, remain intact. Later, a second major reprogramming event occurs in the germ line where paternal and maternal somatic programming is erased from most genes, including imprinted genes. Parent-specific imprints are subsequently imposed in the germ line and DNA methylation across the genome occurs through the action of DNA methyltransferases (for a complete review see Daxinger and Whitelaw, 2012). During these periods of reprogramming, exposure to a challenge that increases or decreases the activity of the epigenomic machinery may lead to alterations in DNA methylation. Embryonic reprogramming suggests that epigenetic modifications to the DNA made in response to drug exposure may be lost, however, there is evidence that some methylation marks escape complete erasure. The best known example is that of imprinted genes, whose methylation marks are retained in the developing embryo (Bartolomei, 2009). In addition there is growing evidence that non-imprinted genes and repetitive genomic elements escape complete loss of methylation patterning following reprogramming events (Lane et al., 2003; Orozco et al., 2014). The retention of genomic methylation patterns in sperm of exposed parents and brains of offspring may occur following generational stress (Franklin et al., 2010) or drug-exposure (Govorko et al., 2012).

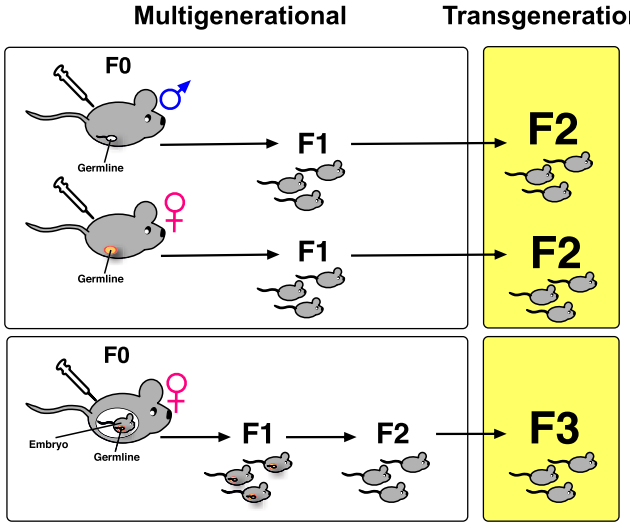

Fig. 1. Multigenerational and Transgenerational Phenotypes Following Drug Exposure. Paternal or maternal exposure paradigms are used as a model for inheritance in rodents. F0 males or females exposed to drug prior to mating produce F1 offspring with multigenerational phenotypes and F2 offspring with transgenerational phenotypes (*top*). F0 females exposed to drug during pregnancy produce F1 and F2 offspring with multigenerational phenotypes and F3 offspring with transgenerational phenotypes (*bottom*).

Histone acetylation and methylation, although not as well described as DNA methylation, may also be subject to epigenetic mechanisms of modulation by drug exposure. Although the vast majority of histones are replaced by protamines during spermatogenesis, not all histones are lost and sperm DNA retention within histones has been discovered in both humans and mice (Bench et al., 1996; Gatewood et al., 1987; Hammoud et al., 2009). Furthermore, DNA methylation retention in sperm may be due to the association of DNA to histones. There is an enrichment of histone bound sperm DNA at loci of imprinted genes and developmentally important genes that retain methylation marks in the embryo (Hammoud et al., 2009; Wykes and Krawetz, 2003).

Histone variants as well as the presence of histone methyltransferases have been identified in mature oocytes and differential acetylation and methylation has been implicated in oocyte development (Wang et al., 2014a). In addition, histone marks in the oocyte can be transmitted across generations (Gaydos et al., 2014). Therefore the genome content in sperm and oocytes along with chemical modifications are potential carriers of epigenetic information.

5. Inheritance of drug exposure

Substance abuse is influenced by a genetic component (Kendler et al., 2003; Tsuang et al., 1998), with heritability estimates ranging from 45% to 79% (Agrawal and Lynskey, 2006; Kendler et al., 2003; Tsuang et al., 2001). Individuals with a family history of drug abuse have an 8-fold increase in the likelihood of drug use, suggesting familial transmission of substance abuse disorders (Merikangas et al., 1998). The heritability of substance abuse varies depending on drug class, although polymorphisms altering gene expression and function are associated with drug-taking susceptibility and resilience across all substances of abuse (Goldman et al., 2005).

While genetics plays a role in variation of drug initiation and dependence, progress in identifying genetic factors that are responsible for addiction risk across most drug classes has been modest. The contribution of other factors that are involved in addiction susceptibility must account for the prevalence of drug use

disorders. Indeed, in addition to the genetic information carried in the DNA sequence; offspring also receive epigenetic information from parents in the form of chemical modifications of DNA and associated histones. These modifications (see Box 1) can significantly alter gene expression. In the following sections, we will discuss the specific epigenetic modifications (if known) that are associated with multi- and transgenerational inheritance of phenotype for each drug class.

5.1. Alcohol

The effects of alcohol are mediated through several neurotransmitter systems. Alcohol directly reduces glutamate signaling at the N-methyl-D-aspartate (NMDA) receptor and enhances gamma amino butyric acid receptor (GABA) signaling at the $GABA_A$ receptor. In addition alcohol mediates increased dopamine and opioid release. Genetic variation in *GABRA2*, the gene encoding for the alpha 2 subunit of the $GABA_A$ receptor has been associated with alcohol dependence in humans and alcohol response in animals (Demers et al., 2014). In addition, polymorphisms in alcohol dehydrogenase and aldehyde dehydrogenase are associated with alcohol consumption; both genes encode for enzymes responsible for metabolism of alcohol. Interestingly, variation in alcohol dehydrogenase and dopamine receptor or dopamine transporter genes has been found to epistatically influence alcohol dependence (reviewed in Demers et al., 2014). Finally, there is evidence to suggest that genetic variation in serotonin, opioid, and other neuromodulatory systems can affect alcohol use (reviewed in Kreek et al., 2004).

Clinical reports have provided evidence of inherited drug use behavior in individuals whose parents engage in alcohol consumption. Episodic drinking in both parents and heavy paternal drinking is correlated with early onset of drinking and amount of alcohol consumed in offspring (Vermeulen-Smit et al., 2012). The heritability of alcohol dependence is well-studied with estimates ranging from 40% to 70% (Agrawal and Lynskey, 2008; Enoch and Goldman, 1999; Nieratschker et al., 2013). Thus, genetics explains some but not all alterations in neurochemical response to alcohol.

The behavioral effects of alcohol are well studied as well as the consequences of parental alcohol use on fetal development. Specifically, F0 mothers exposed to alcohol during pregnancy produce offspring with neurodevelopmental deficits (Lam et al., 2000) and alcohol-exposed F0 fathers produce several generations of offspring with developmental and physiological deficits (Friedler, 1996). Inheritance models in animals have identified changes in reward behavior, neurochemical signaling, and brain morphology in F1 offspring of F0 fathers exposed to alcohol (summarized below and in Table 1). However, to date, no work has described the neurobehavioral effects on offspring of F0 alcohol-exposure in mothers prior to conception.

5.1.1. Behavioral, neurochemical, and structural changes in offspring of alcohol-exposed parents

Paternal alcohol exposure alters reward directed behaviors in offspring. Specifically, when F0 fathers are exposed to alcohol, male F1 offspring show increased sensitivity to amphetamine (Abel, 1993) and alcohol (Finegersh and Homanics, 2014). As amphetamine and alcohol modulate dopamine signaling in the mesolimbic pathway, these results suggest that alcohol mediates functional changes in neuronal dopamine response across generations. However, alcohol exposed F0 fathers appear to pass different phenotypes to their sons and daughters. F0 alcohol-exposed fathers produce F1 female offspring with increased sensitivity to amphetamine (Abel, 1993) but not alcohol (Finegersh and Homanics, 2014).

In addition to drug response, cognitive behaviors implicated in drug abuse and dependence have been assayed in F1 offspring of F0 fathers exposed to alcohol (Abel and Tan, 1988; Kim et al., 2014; Meek et al., 2007; Wozniak et al., 1991). Decreased fear and increased aggression is found in F1 mice from F0 fathers exposed to acute alcohol prior to mating (Meek et al., 2007). In addition, F0 alcohol-exposed fathers produce F1 male offspring with deficits in attention and increased impulsivity (Kim et al., 2014). Learning and memory in F1 animals are differentially affected by F0 alcohol exposure based on the cognitive task being assayed and the animal species tested. F0 male mice exposed to alcohol produce F1 offspring with enhanced passive avoidance learning (Abel and Lee, 1988) while, in rats, female F1 offspring of F0 alcohol-exposed fathers perform worse in a passive avoidance learning task (Abel and Tan, 1988). However, learning deficits are found in both mice and rat F1 offspring of F0 alcohol-exposed father in working memory (choice point task in the T-maze) (Abel and Lee, 1988) and spatial learning (radial arm maze) (Wozniak et al., 1991).

Alcohol also mediates changes in offspring within several neurotransmitter systems that include dopamine, acetylcholine, and opioids. Alcohol-exposed fathers produced F1 offspring with decreased dopamine transporter (DAT) expression in the cortex and striatum (Kim et al., 2014) and hyperactivity in F1 male offspring of F0 alcohol-exposed fathers is attenuated by increased levels of acetylcholine through administration of the cholinesterase inhibitor physostigmine (Abel, 1994). In addition, alcohol exposure in F0 male rats during puberty decreases beta-endorphin, a primary endogenous ligand for opioid receptors, in the hypothalamus of F1 male offspring (Cicero et al., 1990) and F0 females exposed to alcohol during pregnancy produce F1 males with decreased POMC (the gene that codes for beta-endorphin and melanocortin) expression in the arcuate nucleus. In addition, this effect is transmitted to F2 and F3 offspring through the paternal line (Govorko et al., 2012). Endocrine signaling from the hypothalamus is essential for stress response and plays a critical role in the acquisition of drug addiction (Koob and Volkow, 2009). Thus, these neurochemical changes may establish a multi- and transgenerational vulnerability to drug use in offspring of alcohol-exposed fathers.

In addition to behavioral and neurochemical phenotypes, F0 fathers exposed to alcohol produce F1 offspring with altered brain morphology. Cortical thickening is found in F1 offspring of F0 fathers exposed to alcohol before or during mating (Jamerson et al., 2004). Specifically, alterations in thickness are found in cortical sections I and V. Layer I receives projections from pyramidal cells of other cortical layers and layer V contains pyramidal cells. Consequently, the structural effects on these layers suggest alterations in pyramidal cell density and construction of the cortex. However, in another study no differences were found in pyramidal cell density of areas CA1 and CA3 in the hippocampus of offspring derived from F0 fathers exposed to alcohol (Zajac et al., 1989). Therefore, additional research must be done to better understand the change in neuronal morphology in F1 offspring of F0 fathers exposed to alcohol.

5.1.2. Alcohol exposure and changes to the epigenome

Alcohol exposure produces a variety of epigenetic modifications and related gene expression changes in brain reward regions (Robison and Nestler, 2011). Epigenetic modifications have been identified within the F0 generation of animals exposed to alcohol. In addition, evidence of multi- and transgenerational epigenetic changes in animals will be discussed.

Chronic alcohol use upregulates long-term repeat (LTR) transposons through a decrease in methylation (Ponomarev et al., 2012). LTR transposons are transposable elements (TEs) that are typically silenced by the presence of DNA methylation

Table 1
Multigenerational and Transgenerational phenotypes of F0 drug exposure.

	Drug	F0 exposure	Generation affected	Sex of offspring affected	Phenotype	Species	Source
Behavior	Alcohol	Paternal	F1	Both	Increased sensitivity to amphetamine	Rat	Abel, 1993
		Paternal	F1	Male	Reduced alcohol intake and increased behavioral response to alcohol	Mouse	Finegersh and Homanics, 2014
		Paternal	F1	Both	Decreased fear and increased aggression	Mouse	Meek et al., 2007
		Paternal	F1	Male	Decreased attention and increased impulsivity	Mouse	Kim et al., 2014
		Paternal	F1	Both	Enhanced passive avoidance learning and deficits in T-maze	Mouse	Abel and Lee, 1988
		Paternal	F1	Female	Deficits in passive avoidance learning	Rat	Abel and Tan, 1988
		Paternal	F1	Both	Deficits in radial arm maze learning	Rat	Wozniak et al., 1991
		Paternal	F1	Male	Hyperactivity attenuated by physostigmine treatment	Rat	Abel, 1994
	Morphine	Maternal	F1	Both	Increased morphine sensitization (locomotor)	Rat	Byrnes, 2005
		Maternal	F1, F2	Male	Decreased locomotor response to D1/D2 agonist	Rat	Byrnes et al., 2013
	Cocaine	Paternal	F1	Male	Resilience to cocaine self-administration	Rat	Vassoler et al., 2013b
		Maternal	F1	Male	Increased sensitivity to cocaine	Rat	Sasaki et al., 2014
		Paternal	F1	Female	Learning deficits	Mouse	He et al., 2006
		Paternal	F1	Both	Increase in depression-like phenotype, no change in anxiety or learning and memory	Mouse	Killinger et al., 2012
	CB1/CB2 agonist (WIN 55, 212-12)	Maternal	F1	Male	Increased morphine CPP	Rat	Byrnes et al., 2012
		Maternal	F1	Female	Increased morphine sensitization (locomotor)	Rat	Vassoler et al., 2013a
	Δ9-THC	Both parents exposed and mated together	F1	Both	Increased heroin self-administration and enhanced heroin withdrawal	Rat	Szutorisz et al., 2014
	Nicotine	Maternal	F1, F2, F3	Both	Hyperactive, decreased attention	Mouse	Zhu et al., 2014
Neurochemical	Alcohol	Paternal	F1	Both	Decreased DAT in cortex and striatum	Mouse	Kim et al., 2014
		Paternal	F1	Male	Decreased beta-endorphin in hypothalamus	Rat	Cicero et al., 1990
		Maternal	F1, F2, F3	Both	Decreased POMC expression in arcuate nucleus	Rat	Govorko et al., 2012
	Morphine	Paternal	F1	Female	Increased beta-endorphin in hypothalamus	Rat	Cicero et al., 1991
		Maternal	F1	Male	Increased kappa-opioid receptor in response to D1/D2 agonist in nucleus accumbens	Rat	Byrnes et al., 2013
		Maternal	F1, F2	Male	Increased D2 receptor in response to D1/D2 agonist in nucleus accumbens	Rat	Byrnes et al., 2013
		Maternal	F1, F2	Male	Decreased quinpirole induced cortiscosterone	Rat	Byrnes et al., 2013
	Cocaine	Maternal	F1	Male	Increased D1 receptor in medial prefrontal cortex	Rat	Sasaki et al., 2014
	Δ9-THC	Maternal	F1	Female	Increased Oprm1 in nucleus accumbens	Rat	Vassoler et al., 2013a
		Both parents exposed and mated together	F1	Both	Decreased expression of cannabinoid, dopamine, and glutamate receptors, reduced NMDA binding and enhanced LTD in the dorsal striatum	Rat	Szutorisz et al., 2014
Structural Physiological	Alcohol	Paternal	F1	Both	Cortical thickening	Rat	Jamerson et al., 2004
		Paternal	F1	Both	No change in pyramidal cells of hippocampus	Rat	Zajac et al., 1989
	Morphine	Paternal	F1	Both	Decreased hippocampal LTP	Rat	Sarkaki et al., 2008
		Maternal	F1	Both	Decreased hippocampal LTD	Rat	Sarkaki et al., 2008
		Paternal	F2	Both	Decreased synaptophysin	Rat	Vyssotski et al., 2011

and have been increasingly implicated in epigenomic regulation (Pray, 2008; Slotkin and Martienssen, 2007). Interestingly methylation marks on TEs are stable in gametes and strongly implicated in inheritance (Whitelaw et al., 1999). In addition, chronic alcohol use upregulates gene expression networks composed of gene sequences rich in CG repeats and down-regulates gene expression networks composed of low CG repeats (Ponomarev et al., 2012). CG-rich genes involved in synaptic transmission, specifically glutamatergic transmission, are heavily upregulated in the cortex of alcoholics (Ponomarev et al., 2012). Alcohol decreases the activity of DNA methyltransferase 1 (DNMT1), which donates methyl groups to nucleotide residues, thereby providing a possible mechanism for this regulation (Ponomarev et al., 2012). However, alcohol may also mediate reduced methylation through its regulation of the methyl donor cycle. Rats exposed to chronic ethanol have reduced production of the methyl donor S-adenosylmethionine (SAM) and hypo-methylation of the entire genome in the liver (Lu and Mato, 2005). Although the same effect has not been found in neurons or gametes, alcohol may mediate methylation changes through similar signaling.

Imprinted genes appear especially vulnerable to epigenetic modification mediated by alcohol exposure. Demethylation is detected at the locus of imprinted gene *H19* in gametes of male alcoholics (Ouko et al., 2009). In addition, changes in *H19* methylation have been identified in rodent models of multigenerational alcohol exposure. Specifically, F0 mothers exposed to alcohol during pregnancy produce F1 male offspring with decreased

methylation at CpGs in *H19* in sperm and F2 male offspring with deceased methylation at *H19* in brains (Stouder et al., 2011). Hypomethylation at *H19* produces alterations in strict imprinting patterns (Knezovich and Ramsay, 2012; Stouder et al., 2011), that normally escape complete methylation erasure during steps of developmental reprogramming. While this suggests transmission of aberrant methylation from F1 sperm to F2 somatic cells in offspring, a second study found hypomethylation at *H19* in F1 offspring but no alteration in sperm of alcohol-exposed F0 fathers (Knezovich and Ramsay, 2012).

Alcohol increases and decreases DNA methylation in non-imprinted genes and both multigenerational and transgenerational epigenetic inheritance of such changes have been found. F0 alcohol exposure decreases methylation at the brain derived neurotrophic factor (*BDNF)* exon IV promoter in the sperm of exposed males. This reduction of methylation is also measured at the exon IV promoter of BDNF in the ventral tegmental area (VTA) of F1 offspring (Finegersh and Homanics, 2014). Consequently, associated with the decrease in *BDNF* methylation, increased exon IV transcripts are found in the VTA (Finegersh and Homanics, 2014). In contrast, increased methylation is found in the promoter region of *DAT* and decreased mRNA and protein expression of the dopamine transporter in the cortex and striatum of F1 offspring of F0 fathers exposed to alcohol (Kim et al., 2014). Alcohol-mediated decrease in DNMT1, as was discussed with alcohol-mediated regulation of LTRs and CpG dense regions, is thought to also mediate hypermethylation of *DAT* in the cortex and striatum of F1 offspring (Kim et al., 2014). Finally, F0 females exposed to alcohol during pregnancy produce F1 male offspring and F2 and F3 offspring with increased POMC methylation in the arcuate nucleus. Interestingly, F1 sperm also has an increase in POMC methylation (Govorko et al., 2012). Therefore alcohol produces differential methylation changes depending on the gene target.

Alcohol can also mediate chromatin remodeling in the animal exposed though histone methylation and acetylation. Upregulation of CG-rich genes following alcohol exposure is correlated with increased levels of trimethylation of histone 3 at lysine 4 (H3K4me3) in the promoters of the same genes within a generation (Ponomarev et al., 2012). In addition, some of the genes that are upregulated promote trimethylation at histone 3 which therefore increases H3K4me3 (Ponomarev et al., 2012). This suggests an interaction between alcohol-mediated DNA methylation and chromatin remodeling. In addition to methylation changes on the histone, genome wide histone acetylation is increased following alcohol administration (Ghezzi et al., 2013). In a rat model of alcohol addiction, decreased histone deacetylase (HDAC) activity and an increase in the acetylation of histones H3 and H4 is found in the amygdala (Pandey et al., 2008). Thus alcohol may change the expression of genes through histone methylation and acetylation within the animal being exposed, however unlike DNA methylation, no studies have been conducted to determine if these changes are also found in offspring.

5.1.3. Gametes and the effects of alcohol

Alcohol exposure is detrimental to mammalian reproduction in both males and females. The oocyte is sensitive to the effects of alcohol; parthenogenic activation occurs in oocytes bathed in alcohol through intracellular oscillations of free calcium ions (Cuthbertson, 1983). Thus, in female animals alcohol use affects puberty, disrupts menstrual cycling, and alters reproductive function (Emanuele et al., 2002). In addition, chronic alcohol consumption has detrimental effects on male reproductive hormones and sperm quality (Muthusami and Chinnaswamy, 2005). While a direct effect of alcohol on gametic gene expression has not been found, direct or indirect exposure of the gametes to alcohol does decrease fertility. Therefore, it is confirmed that gametes are receptive to the effects of alcohol exposure.

5.2. Opioids

Opioids such as morphine and heroin are powerful analgesics that act on membrane bound G_i-protein coupled receptors; mu-, kappa- and delta. Opioid receptors are distributed throughout the central nervous system and mediate both the rewarding and analgesic properties of this drug class. Genetic vulnerability for opiate abuse is between 43% and 60% (Ho et al., 2010). The most thoroughly studied genetic association being the A118G single nucleotide polymorphism in the mu opioid receptor gene, *OPRM1*. A complex relationship has been characterized between the A118G polymorphism and the prevalence of opioid abuse in patients (Dick and Agrawal, 2008). However, genetics does not completely explain the inheritance of altered phenotypes in several generations of offspring following initial drug exposure. Recent work has shown that altered morphine response is transmittable to offspring of morphine exposed parents (Byrnes, 2005; Byrnes et al., 2013). Physiological and structural changes in the brains of offspring of morphine-exposed parents have also been characterized (Byrnes et al., 2013; Cicero et al., 1991; Sarkaki et al., 2008; Vyssotski, 2011) (Table 1).

5.2.1. Behavioral, neurochemical, and structural changes in offspring of opioid-exposed parents

Opioid exposure in F0 parents changes offspring response to drug administration (Byrnes, 2005; Byrnes et al., 2013). Female F0 rats exposed to morphine during adolescence give birth to offspring with altered development of morphine and dopamine-mediated locomotor sensitization. Both male and female F1 offspring from female F0 rats exposed to morphine prior to pregnancy have significantly increased locomotor activity in response to morphine (Byrnes, 2005). In addition, male F1 offspring show attenuated locomotor sensitization to the dopamine receptor 1 and 2 (D1/D2) agonist quinpirole (Byrnes et al., 2013). Interestingly, this phenotype is transmitted through the maternal line as F1 female rats derived from F0 morphine exposure also produce F2 male offspring with attenuated locomotor sensitization to the D1/D2 agonist quinpirole (Byrnes et al., 2013)

Neuroadaptations in dopamine signaling may explain why response to drug challenges are different in F1 male and female offspring and male F2 offspring of morphine-exposed F0 mothers. F0 morphine-exposed mothers produce male F1 offspring with increased D2 receptor mRNA expression in the nucleus accumbens (NAc) in response to chronic D1/D2 agonist exposure. The same changes are found in F2 male offspring of F1 females from F0 morphine-exposed mothers (Byrnes et al., 2013). Therefore, dopamine response is altered due to parent drug history and the trajectory of offspring drug response is shaped accordingly due to these changes in dopamine reward circuitry which are implicated in drug addiction (Wise, 1998).

The endogenous opioid system is also altered in F1 offspring of F0 morphine-exposed males and females. Specifically, morphine-exposed fathers produce F1 female offspring with increased beta-endorphin in the hypothalamus and increased circulating serum cortiscosterone levels (Cicero et al., 1991). Similar changes have been found in additional generations. Morphine-exposed F0 mothers produce male F1 and F2 offspring (through the maternal line) with decreased quinpirole induced cortiscosterone (Byrnes et al., 2013). Dopamine and opioid interactions are also altered in offspring of morphine-exposed F0 mothers. Female F0 rats exposed to morphine during adolescence produce male F1 offspring with increased kappa opioid receptor mRNA expression in the NAc

following chronic D1/D2 agonist exposure (Byrnes et al., 2013). Thus, multi- and transgenerational effects of morphine exposure can be seen as changes in opioid neuroadaptations in response to D1/D2 agonist exposure in offspring.

Physiological changes in offspring of morphine-exposed parents have also been identified. Alterations in hippocampal synaptic plasticity have been found in the F1 offspring of F0 morphine-exposed males and females mated together (Sarkaki et al., 2008). Synaptic plasticity is essential to the process of learning and memory as well as learned behaviors that include drug abuse (Hyman et al., 2006). Long-term potentiation (LTP) and long-term depression (LTD) reflect increases and decreases in synaptic strength, respectively. Decreased LTP is found in the hippocampus of F1 offspring of F0 morphine treated fathers. In contrast, F0 morphine-exposed mothers produce F1 offspring with decreased LTD in the hippocampus but intact hippocampal LTP (Sarkaki et al., 2008). These findings suggest that morphine mediates physiological changes that result in impaired synaptic plasticity in the hippocampus of F1 offspring.

Finally, transgenerational effects on cellular morphology have been observed following morphine exposure, suggesting parental morphine use can change neuronal structure. Decreased synaptophysin is found in the brains of F2 offspring derived from morphine-exposed F0 male rats (Vyssotski, 2011). As synaptophysin expression is associated with the density of nerve terminals, decreased synaptic connections exist in the F2 male brains of F0 morphine-exposed fathers.

Together these studies demonstrate that morphine exposure can impact behavior, biochemical signaling, synaptic plasticity, and neuronal structure across multiple generations. While behavioral and biochemical effects are observed in F1 generations, extension of modifications in cellular morphology out to the F2 offspring indicates a transgenerational inheritance of morphine-exposure.

5.2.2. Opioid exposure and changes to the epigenome

To date no definitive modification to the epigenome has been identified that could explain transgenerational inheritance of morphine exposure, however there have been reports of epigenetic modifications following morphine exposure within a single generation. For example, histone modifications have been measured in the NAc and other brain regions following exposure to morphine in animals (Maze and Nestler, 2011; Sun et al., 2012). Morphine reward and sensitization in mice following chronic morphine administration has been correlated with global levels of histone 3 lysine 9 dimethylation (H3K9me2) in the NAc (Sun et al., 2012). Because H3k9me2 is typically associated with repression of transcription, decreased H3k9me2 results in increased gene expression (Rice and Allis, 2001), and this gene expression may mediate the rewarding effects of morphine.

Histone methylation of long-interspersed nuclear (LINE-1) retrotransposons is decreased in the NAc of mice following direct injection of morphine to the area (Sun et al., 2012). LINE-1 elements are repetitive DNA sequences that make up a large portion of genomic DNA (~20%) (Prak and Kazazian, 2000). The mobilization, integration, and regulation of these elements have been characterized in several brain regions (Singer et al., 2010) and alterations in LINE-1 expression through epigenetic mechanisms have been implicated in drug addiction within the user (Maze et al., 2011; Prak and Kazazian, 2000). Interestingly, transgene mobilization and expression through epigenomic release from silencing were some of the first epigenetic changes to be implicated in transgenerational inheritance (Daxinger and Whitelaw, 2012). While LINE-1 expression is associated with morphine use in somatic cells, it is not known if LINE-1 expression is altered in the germ cells of F0 morphine-exposed parents and if this is maintained in the brains and gametes of F1 offspring or future generations.

Histone acetylation is also altered in response to morphine exposure. In rats, exposure to morphine increases acetylation of histone H3 lysine 14 (aceH3K14) in the NAc (Sheng et al., 2011) and basolateral amygdala (Wang et al., 2014b), a region critically involved in stimulus–reward associations (Everitt et al., 1999). Histone acetylation is associated with transcriptional activation (see Box 1). While these modifications have been found within a generation, there is no evidence of altered histone acetylation in offspring following parental morphine exposure.

In addition to methylation and acetylation modifications of histones, morphine exposure induces methylation changes on DNA directly (Nielsen et al., 2008; Chorbov et al., 2011; Doehring et al., 2013; Trivedi et al., 2014). Morphine modulates cellular oxidative stress and methyl group donation through the antioxidant glutathione and the methyl donor SAM, respectively (Trivedi et al., 2014). Consequently, as the redox state of the cell impacts methylation events, DNA methylation changes can occur following morphine exposure. Just as LINE-1 seems to be vulnerable to histone methylation changes following morphine exposure, the transposable element exhibits decreases in methylation after morphine administration. Hypomethylation at LINE-1 retrotransposons has been identified in leukocytes of chronic heroin users (Doehring et al., 2013) and in neuronal cell lines (Trivedi et al., 2014); consequently, LINE-1 mRNA expression increases (Trivedi et al., 2014). In contrast, increased CpG methylation has been reported in lymphocytes, blood, and sperm of chronic heroin users in the promoter region of the *OPRM1* gene (Chorbov et al., 2011; Nielsen et al., 2008). Thus, morphine administration can lead to both increased and decreased DNA methylation in a gene-dependent manner in the individual user. To date no studies have determined if these chemical modifications are inherited by offspring.

5.2.3. Gametes and opioid receptors

Localization of the mu opioid receptor as well as endogenous expression of beta-endorphin in the male reproductive tract suggests that paternal gametes are receptive to endogenous and exogenous opioids (Albrizio et al., 2006). Mu-, kappa- and delta opioid receptors are present in oocytes, most likely for mediating oocyte maturation (Agirregoitia et al., 2012). Thus the presence of opioid receptors on gametes not only maintains proper function and development, but may mediate transgenerational inheritance.

5.3. Cocaine

Cocaine increases dopamine by binding to the dopamine transporter and blocking the reuptake of dopamine into the presynaptic cell. The resulting increase in dopamine in the NAc is associated with the rewarding effects of cocaine (Wise, 1998). Cocaine use heritability is 44% based on Vietnam Era Twin Registry studies (Tsuang et al., 1996). Inter-individual differences such as baseline dopamine signaling (Volkow et al., 1999) and allelic variation in genes including dynorphin and dopamine beta-hydroxylase (DBH) (LaForge et al., 2000) have been correlated with risk of cocaine use and abuse. However, as genetics accounts for less than 50% of heritability for cocaine use, other factors likely contribute to cocaine use and abuse across generations. Current research has identified multigenerational effects on behavior, neurochemical signaling (Table 1), and the epigenome following cocaine administration in animal studies.

5.3.1. Behavioral and neurochemical changes in offspring of cocaine-exposed parents

In utero exposure to cocaine is well-studied; in animals cocaine exposed offspring show behavioral, biochemical, and structural alterations in the brain (Vassoler et al., 2014). While, there are currently no reports on the transgenerational inheritance of cocaine exposure there is evidence of multigenerational effects.

Studies employing F0 parental cocaine exposure have found changes in behavior in F1 male and female offspring. For example, F1 male offspring of F0 cocaine-exposed fathers find cocaine less rewarding; male rats acquire self-administration more slowly and administer less drug, while F1 female rats are not affected (Vassoler et al., 2013b). In addition, male F1 offspring of F0 mothers exposed to cocaine prior to pregnancy are more sensitive to the psychomotor effects of cocaine administration (Sasaki et al., 2014). However, contrasting results have been reported for behaviors that may influence drug abuse in offspring derived from paternal cocaine exposure; some have found learning deficits in female F1 offspring of F0 fathers exposed to cocaine (He et al., 2006) while others report no learning effects in either male or female F1 offspring of F0 cocaine-exposed fathers (Killinger et al., 2012). While these results demonstrate some multigenerational effects of cocaine exposure, examination of these phenotypes in the F2 generation will determine transgenerational inheritance.

In addition to changes in behavior, alterations in neurotransmitter systems have been found in F1 offspring of F0 cocaine-exposed mothers. Specifically, F0 cocaine-exposed mothers produce F1 male offspring with increased D1 receptor expression in the medial prefrontal cortex (mPFC) (Sasaki et al., 2014). An increase in the number of dopamine receptors in the mPFC could cause enhanced cocaine response due to the ability of these receptors to activate the mesocortical dopamine pathway and activity at the D1 receptor mediates the psychomotor response to cocaine in rodents (Steketee and Kalivas, 2011; Xu et al., 1994).

5.3.2. Cocaine exposure and changes to the epigenome

Multigenerational changes in histone 3 acetylation (AcH3), commonly associated with chromatin remodeling and increased transcription of target genes has been identified in response to cocaine exposure. F0 fathers that self-administer cocaine have increased AcH3 in the testes and greater AcH3 association with exon IV of BDNF in sperm (Vassoler et al., 2013b). F1 offspring of these fathers also show increased AcH3 association with exon IV of BDNF and increased transcription of BDNF in the mPFC (Vassoler et al., 2013b). BDNF and its modulation through chromatin remodeling has been implicated in both chronic and generational cocaine exposure (Kumar et al., 2005; Sasaki et al., 2014; Vassoler et al., 2013b). Functional antagonism of increased BDNF in the F1 male offspring of F0 cocaine-exposed fathers increases cocaine self-administration to that of control animals (Vassoler et al., 2013b). Thus epigenetic changes can occur across generations and suggest that chromatin remodeling in response to drug use may be an important mechanism of inheritance and cocaine response. Additional evidence supports that drug reinforcement may converge on a common epigenetic pathway. Like morphine (Sun et al., 2012), direct cocaine exposure in mice reduces H3K9me2 in the NAc and these changes mediate cocaine preference and cellular plasticity (Maze et al., 2010). However, multigenerational or transgenerational inheritance of this modification has not been examined.

Epigenomic machinery, specifically methyl-CpG binding protein (MeCP2) and DNA methyltransferase 3a (DNMT3a), are altered within a generation following exposure to cocaine. Increased expression of MeCP2 is found after cocaine self-administration (Host et al., 2011); MeCP2 acts as a repressor or activator of transcription when it binds to the promoter region of a target gene (Chahrour et al., 2008). MeCP2 is involved in learning and memory and therefore may be implicated in drug abuse behaviors (Feng and Nestler, 2010). Cocaine stimulation of MeCP2 also represses the transcription of two small non-coding RNAs, miR-212 and miR-312 (Im et al., 2010). Decreased transcription of these miRNAs releases BDNF from miRNA transcriptional repression resulting in increased BDNF (Im et al., 2010) which has been shown to mediate cocaine use (Sadri-Vakili et al., 2010).

DNA methylation can be altered following cocaine exposure through regulation of DNA methyltransferase 3a (DNMT3a) (LaPlant et al., 2010). While, DNMT3a regulates cocaine response it is also important in modulating *de novo* methylation of differentially methylated regions of paternal imprinted genes and some repetitive elements in male germ cell development. For example, DNMT3a is responsible for methylation of both the imprinted gene *H19* and the repetitive element LINE-1 (Kato et al., 2007). Similar to morphine, cocaine alters levels of the antioxidant glutathione that can lead to impaired methylation of the genome (Lee et al., 2001). Thus, cocaine mediates changes in the activity of molecules responsible for the methylation status of DNA. However, it remains to be determined if these changes are found in F1 and subsequent generations of offspring.

Although little has been published on transgenerational changes in DNA methylation following cocaine exposure, DNA methylation is altered in F1 offspring with *in utero* exposure to cocaine. F0 mothers exposed to cocaine during pregnancy produce F1 offspring with altered global patterns of hippocampal DNA methylation. These changes seem to be functionally significant as they are accompanied by corresponding alterations in gene transcription (Novikova et al., 2008).

5.3.3. The effects of cocaine on gametes

Testes contain high affinity binding sites for cocaine (Sweet and Murdock, 1987), exceeded in number only by the brain (Mulé and Misra, 1977). In addition, the presence of DAT protein in sperm has been characterized and dopamine signaling is implicated in sperm viability, mobility, and capacitation (Ramírez et al., 2009). Interestingly, cocaine remains bound to human sperm at traceable levels for up to 24 h, and there is history of concern for cocaine transmission via “piggybacking” on sperm to the oocyte (Cone et al., 1996; Yazigi et al., 1991). Although cocaine presence in early embryonic development is known to be detrimental, the more likely process of transmission of phenotypes associated with cocaine exposure from F0 father to F1 offspring is through the epigenetic mechanisms previously discussed. Thus, changes in DNA methylation, and chromatin state in sperm could be an important mechanism for transgenerational inheritance of behavioral consequences of cocaine exposure. To date, it is unclear if oocytes have binding sites for cocaine and therefore, any maternally-derived effects of cocaine exposure may be due to indirect mechanisms within the oocyte.

5.4. Marijuana

The active component in marijuana, Δ9-tetrahydrocannabinol (Δ9-THC), is responsible for the drug's biological effects. Δ9-THC binds to and activates G_i-coupled cannabinoid type 1 and 2 (CB1 and CB2) receptors throughout the central nervous system. A meta-analysis of cannabis initiation and problematic cannabis use among twins suggest that the heritability of cannabis initiation is 40–48% and chronic problematic use is 51–59% depending on gender (Verweij et al., 2010). However, higher genetic associations for cannabis use have been found in female twin studies (Kendler and Prescott, 1998).

Polymorphic variation in the gene that codes for cannabinoid receptor 1 (*CNR1*) has been implicated in marijuana craving during times of abstinence and withdrawal (Demers et al., 2014). In addition, polymorphic variation in *CYP2C9*, a member of the cytochrome family of enzymes responsible for the metabolism of Δ9-THC, results in greater active metabolite and heightened sensitivity to the sedative effects of marijuana (Sachse-Seeboth et al., 2008). More recently clinical studies suggest an interaction between genetics and epigenetics that alters response to marijuana use. In adolescents homozygous for the catechol-o-methyltransferase (COMT) $Val^{108/158}Met$ polymorphism, methylation at the promoter of this gene is associated with lower risk of marijuana use (van der Knaap et al., 2014). Greater methylation at the promoter of COMT reduces expression of the enzyme. In addition the homozygous Met/Met genotype is associated with decreased enzyme activity. Together these changes result in decreased degradation of COMT substrates such as dopamine. Therefore, individuals with hypermethylation and the polymorphism have increased dopamine levels. Conversely, low brain dopamine results in an attenuated reward system that manifests into anhedonia. It is hypothesized that substance use is an attempt to alleviate this unfavorable anhedonic state (Markou and Koob, 1991; Volkow et al., 2010). Hence, Met/Met individuals with hypermethylation may be less vulnerable to substance abuse due to increases in dopamine. While genetic interactions explain some of the risk for marijuana use, other factors must also affect administration in individuals. Currently, multigenerational effects of cannabinoid exposure have been identified (Table 1).

5.4.1. Behavioral and neurochemical changes in offspring of cannabinoid-exposed parents

Endocannabinoid and opioid systems closely interact as cannabinoid exposure can cause cross-sensitization to the effects of opioids in dopaminergic neurons (Pistis et al., 2004). To date, studies have only examined the consequence of cannabinoid exposure on morphine response in offspring. Response to morphine administration is altered in F1 offspring of mothers exposed to the CB1/CB2 receptor agonist WIN 55, 212-2. Specifically, F0 mothers exposed to WIN 55, 212-2 during adolescence produce male offspring with increased morphine conditioned place preference (Byrnes et al., 2012) and female offspring with increased morphine locomotor sensitization (Vassoler et al., 2013a). Therefore, both drug response and reinforcement is altered in the F1 offspring of WIN 55, 212-2-exposed mothers. In addition, male and female rats exposed to Δ9-THC and then mated together produce F1 offspring that show increased motivation to self-administer heroin as well as enhanced withdrawal behaviors during periods of opioid withdrawal (Szutorisz et al., 2014). Therefore, while cross-sensitization between cannabinoids and opioids has been identified in the individual user (Cadoni et al., 2001), the studies discussed above are the first examples of cross-sensitization of cannabinoids and opioids across a generation for reinforcement, drug response, and self-administration. These results suggest multigenerational effects of cannabinoid use; altered response to morphine and heroin occurred exposure to CB1/CB2 agonist or Δ9-THC. However, no data is available for these phenotypes in the F2 generation. In addition, no studies have examined the effects in F1 or F2 offspring of F0 fathers exposed to cannabinoids.

Multigenerational alterations in gene expression and neurotransmission have been found following exposure to cannabinoids. F0 females exposed to CB1 agonist produce F1 female offspring with increased Oprm1 expression in the NAc (Vassoler et al., 2013a). Male and female rats exposed to Δ9-THC and then mated together produce offspring with decreased expression of cannabinoid, dopamine, and glutamate receptors, reduced NMDA binding and enhanced LTD in the dorsal striatum (Szutorisz et al., 2014). Further analysis of these systems is necessary to determine if any of these phenotypes are inherited in additional generations.

5.4.2. Cannabinoid exposure and changes to the epigenome

There is currently no evidence of multi- or transgenerational epigenetic changes following marijuana exposure. However, activation of the immediate early gene FosB occurs following direct Δ9-THC administration (Fürst et al., 2013; Porcella et al., 1998). Cocaine activation of FosB has been correlated to increased histone 4 acetylation in the promoter region of the gene (Kumar et al., 2005) and Δ9-THC may mediate similar chromatin remodeling and gene activation. Indeed, Δ9-THC causes genome wide changes in histone methylation (Yang et al., 2014) although other histone modifications, including acetylation, have not been thoroughly explored. Changes in DNA methylation have not been examined following exposure to marijuana.

5.4.3. Gametes and cannabinoid receptors

CB1 receptors and the endogenous endocannabinoid anandamide are expressed in the ovaries and uterine endometrium (Bari, 2011). Endocannabinoid signaling has been implicated in oocyte maturation, ovulation, and fertility signals (Battista et al., 2012). In addition, cannabinoid receptor localization has been characterized in testes (Schuel et al., 2002) and sperm (Aquila et al., 2010). Furthermore, anandamide analog administration alters the PI3K/Akt cell survival pathway in sperm (Aquila et al., 2010). Further research is required to identify the direct or indirect epigenetic changes caused by marijuana administration in gametes.

5.5. Nicotine

Nicotine is the active ingredient in tobacco and is responsible for the positive experience associated with tobacco use. Nicotine binds to nicotinic acetylcholine receptors (nAChRs) which are pentameric ligand-gated ion channels (Benowitz, 1999). The relative contribution of genetic influence is 44% for smoking initiation and 75% for nicotine dependence (Vink et al., 2005). Work from human GWAS as well as animal research identified several neurotransmitter systems that may play a role in the likeliness of nicotine use and dependence. Genetic variation in dopamine receptor 2 and 4 (*DRD2* and *DRD4*) have been associated with smoking behavior. In addition, genetic variation in the dopamine transporter gene, *SLC6A3*, has been associated with smoking behavior and is also known to interact in an allele-specific epistatic manner with *DRD2* allele expression. Allelic variation in genes that regulate norepinephrine (*DBH* and *MAOA*) and serotonin (*5HTTLPR* and *TPH*) have also been implicated in nicotine use (reviewed in Lerman and Berrettini, 2003). In addition, polymorphic variation in the gene that codes for choline acetyltransferase (*ChAT*) (Ray et al., 2010) as well as variation in the primary enzyme that metabolizes nicotine *CYP2A6* and gene clusters coding for nicotinic acetylcholine receptor (nAChR) subunits (Demers et al., 2014) have been associated with sensitivity to nicotine, tobacco use, and success for smoking cessation. However, association studies have suggested that genetic predisposition accounts for less than half of the likelihood of smoking initiation and three-fourths of nicotine dependence (Vink et al., 2005). Therefore, environmental influence, including epigenetic modifications, may account for remaining risk. Recent evidence suggests the transgenerational inheritance of behavior and multigenerational neurochemical changes following parental nicotine exposure (Table 1).

5.5.1. Behavioral and neurochemical changes in offspring of nicotine-exposed parents

While no studies have examined drug response and reward behavior in F1 or F2 offspring from F0 nicotine-exposed parents, there is evidence that nicotine exposure can cause multigenerational changes in cognition and dopamine in offspring. Zhu and colleagues found that F1 male and female offspring of F0 mothers exposed to nicotine during pregnancy are hyperactive with decreased attention. In addition, this phenotype is transmitted to F2 offspring and F3 offspring through the maternal line (Zhu et al., 2014). Hyperactivity in the F2 offspring is attenuated by methylphenidate-induced dopamine increase (Zhu et al., 2014), thereby implicating a hypodopaminergic state as the mechanism by which nicotine alters offspring response. Thus, F0 maternal exposure to nicotine during pregnancy produces transgenerational changes in behavior and multigenerational changes in dopamine signaling (Zhu et al., 2014). Studies have examined additional neurochemical changes following exposure to nicotine, however, these are only found in offspring of F0 mothers exposed to nicotine during pregnancy (Zhu et al., 2012; Yochum et al., 2014). To date, no neurochemical changes have been identified in animals not directly exposed to nicotine.

5.5.2. Nicotine exposure and changes to the epigenome

The epigenome is vulnerable to modification by nicotine exposure. Global DNA methylation patterns in leukocytes are similar between non-smoking offspring and non-smoking fathers, while there are differences in methylation if offspring are smokers and fathers are non-smokers (Hillemacher et al., 2008). In addition, nicotine-induced variation in DNA methylation has been identified in several genes implicated in drug abuse. For example, nicotine exposure is associated with DNA methylation changes in the gene coding for monoamine oxidase A (*MAOA*), a key enzyme in the metabolism of dopamine and other monoamines. Decreased methylation of the *MAOA* promoter is found in the blood and lymphoblasts of current smokers (Philibert et al., 2010). This modification may be mechanistically important, as it has been proposed by Zhu and colleagues that nicotine induced hypomethylation of *MAOA* decreases dopamine synthesis in animals exposed to nicotine *in utero* (Philibert et al., 2010; Zhu et al., 2012). Chronic nicotine administration produces an increase in expression of DNMT1, resulting in methylation of glutamate decarboxylase (GAD67), and reduced GAD67 mRNA (Satta et al., 2008). Consequently, changes in GAD67 mRNA can influence cortical GABAergic signaling. In addition, evidence that methylation and expression of GAD67, a gene implicated in the phenotype of patients with schizophrenia, is modulated by chronic nicotine administration suggests that the co-morbidity that exists between the disorder and smoking addiction may have an epigenetic basis. Interestingly, DNA methylation has been associated with multigenerational exposure to nicotine. F0 mothers exposed to nicotine during pregnancy produce F1 offspring with increased methylation at *BDNF* in blood (Toledo Rodriguez et al., 2010) and decreased BDNF mRNA and protein in the frontal cortex (Yochum et al., 2014). As discussed previously, decreases in levels/activity of BDNF has been implicated in the self-administration of drugs including cocaine (Sadri-Vakili et al., 2010), as well as methylphenidate (Cadet et al., 2014) and alcohol (Jeanblanc et al., 2012).

In addition to changes in DNA methylation, nicotine can remodel chromatin through histone modifications. Following nicotine administration in mice there is an increase in the acetylation of histone H3 in the striatum (Levine et al., 2011) and a decrease in H3K9me2 at promoter regions of target genes (Chase and Sharma, 2013). Taken together, these studies suggest that nicotine reduces epigenetic histone marks that promote a restrictive genomic state, thereby opening normally repressed genes to enhanced transcription.

5.5.3. Gametes and nicotinic receptors

Nicotine mediated increase in the production of cholesterol, triglycerides, phospholipids and free fatty acids in the testes can be blocked by administration of the nAChR antagonist mecamylamine suggesting binding sites for both drugs in the testes (Kavitharaj and Vijayammal, 1998). Indeed, the mRNA of subunits that compose the pentameric nAChR (i.e. α7, α9, α3, α5, and β4) are expressed in sperm (Kumar and Meizel, 2005). Furthermore, functional nAChRs have been found in sperm (Kumar and Meizel, 2005). Acetylcholine mediated spikes in calcium through α7 homomeric receptors occurs in the sperm head during the acrosomal reaction (Bray et al., 2005). Finally, nicotine, likely acting on nAChRs has detrimental effects on sperm viability. Maternal smoking has been linked to male offspring infertility through gonadal toxicity (Ratcliffe et al., 1992). While it is well-established that nicotine exposure produces reproductive challenges in females and the early developing embryo (Omotoso et al., 2013), nicotine binding has not been identified in oocytes therefore inheritance through the maternal line may be through indirect effects of nicotine exposure.

6. Conclusion and Perspectives

Although the idea that parental experience can influence behavior in offspring is not new, an understanding of drug-mediated changes to the epigenome may provide an avenue to identify mechanisms of transgenerational inheritance in drug addiction. While stress (Franklin et al., 2010) and diet (McGowan et al., 2008) have long been examined as environmental challenges that impact the behavior and physiology of future offspring, drug abuse remains to be thoroughly examined for the same features. Several instances of multigenerational inheritance has been reported across drug types, however there is a deficit of studies that examine transgenerational inheritance of phenotypes following F0 exposure to drugs of abuse (Table 1). To date, transgenerational phenotypes have only been reported following parental exposure to morphine (Byrnes et al., 2013; Vyssotski, 2011), alcohol (Govorko et al., 2012), and nicotine (Zhu et al., 2014).

This review has examined changes in behavior, neurochemical signaling, and cellular structure found in the F1 and F2 offspring of drug-exposed parents. While some studies have implicated epigenetic inheritance of these phenotypes, fewer have identified epigenetic transgenerational inheritance. In addition, alterations to the epigenome in response to drug-exposure have been discussed but no evidence of a specific mechanism for transgenerational epigenetic inheritance of drug exposure has been determined. While there are some common players that are epigenetically modified following drug exposure, such as BDNF and DNMT1, a complex epigenomic network may be uniquely mediated by different classes of drugs of abuse. Identifying novel changes made to the epigenome in response to drug helps to select candidate genes to examine in future generations. The inheritance of these changes can then be identified and characterization of the functional relevance of epigenetic changes in offspring can be determined.

In translating research, drug exposure and epigenetic regulation of the genome becomes quite complex. Animal studies allow for careful control over the environment and drug exposure variables. Thus, such studies can provide the best evidence of the existence of multigenerational and transgenerational inheritance of drug abuse and the possible epigenetic mechanisms that may underlie these phenotypes. Thus, future studies aimed at identification of functional epigenetic modifications following drug in the somatic and

germ cells of F0, F1, and F2 animals after F0 drug exposure are critical.

The scope of current work in transgenerational drug inheritance should focus on extending observations of behavioral phenotypes to F2 and F3 generations and molecular analysis to include brains of offspring and gametes of parents. With advancements in techniques to evaluate epigenetic changes to the genome the field of drug abuse and inheritance is promising. Future work may start to identify the vulnerability of individuals to epigenomic inheritance of drug abuse and provide evidence for mechanisms of genomic regulation through drug exposure.

Acknowledgments

This work was supported by the National Institutes of Health National Institute on Drug Abuse [Grants T32-DA28874 and R01-DA033646].

References

Abel, E.L., 1993. Paternal alcohol exposure and hyperactivity in rat offspring: effects of amphetamine. Neurotoxicol. Teratol. 15, 445–449.
Abel, E.L., 1994. Effects of physostigmine on male offspring sired by alcohol-treated fathers. Alcohol. Clin. Exp. Res. 18, 648–652.
Abel, E.L., Lee, J.A., 1988. Paternal alcohol exposure affects offspring behavior but not body or organ weights in mice. Alcohol. Clin. Exp. Res. 12, 349–355.
Abel, E.L., Tan, S.E., 1988. Effects of paternal alcohol consumption on pregnancy outcome in rats. Neurotoxicol. Teratol. 10, 187–192.
Agirregoitia, E., Peralta, L., Mendoza, R., Expósito, A., Ereño, E.D., Matorras, R., Agirregoitia, N., 2012. Expression and localization of opioid receptors during the maturation of human oocytes. Reprod. Biomed. Online 24, 550–557.
Agrawal, A., Lynskey, M.T., 2006. The genetic epidemiology of cannabis use, abuse and dependence. Addiction 101, 801–812.
Agrawal, A., Lynskey, M.T., 2008. Are there genetic influences on addiction: evidence from family, adoption and twin studies. Addiction 103, 1069–1081.
Albrizio, M., Guaricci, A.C., Calamita, G., Zarrilli, A., Minoia, P., 2006. Expression and immunolocalization of the mu-opioid receptor in human sperm cells. Fertil. Steril. 86, 1776–1779.
Aquila, S., Guido, C., Santoro, A., Perrotta, I., Laezza, C., Bifulco, M., Sebastiano, A., 2010. Human sperm anatomy: ultrastructural localization of the cannabinoid1 receptor and a potential role of anandamide in sperm survival and acrosome reaction. Anat. Rec. Hob. 293, 298–309.
Bari, M., 2011. The manifold actions of endocannabinoids on female and male reproductive events. Front. Biosci. 16, 498.
Bartolomei, M.S., 2009. Genomic imprinting: employing and avoiding epigenetic processes. Genes Dev. 23, 2124–2133.
Battista, N., Meccariello, R., Cobellis, G., Fasano, S., Di Tommaso, M., Pirazzi, V., Konje, J.C., Pierantoni, R., Maccarrone, M., 2012. The role of endocannabinoids in gonadal function and fertility along the evolutionary axis. Mol. Cell. Endocrinol. 355, 1–14.
Bench, G.S., Friz, A.M., Corzett, M.H., Morse, D.H., Balhorn, R., 1996. DNA and total protamine masses in individual sperm from fertile mammalian subjects. Cytometry 23, 263–271.
Benowitz, N.L., 1999. Nicotine addiction. Prim. Care Clin. Office Pract. 26, 611–631.
Bird, A., 2002. DNA methylation patterns and epigenetic memory. Genes Dev. 16, 6–21.
Bird, A., 2007. Perceptions of epigenetics. Nature 447, 396–398.
Bray, C., Son, J.-H., Kumar, P., Meizel, S., 2005. Mice deficient in CHRNA7, a subunit of the nicotinic acetylcholine receptor, produce sperm with impaired motility. Biol. Reprod. 73, 807–814.
Byrnes, E.M., 2005. Transgenerational consequences of adolescent morphine exposure in female rats: effects on anxiety-like behaviors and morphine sensitization in adult offspring. Psychopharmacology 182, 537–544.
Byrnes, J.J., Johnson, N.L., Schenk, M.E., Byrnes, E.M., 2012. Cannabinoid exposure in adolescent female rats induces transgenerational effects on morphine conditioned place preference in male offspring. J. Psychopharmacol. 26, 1348–1354.
Byrnes, J.J., Johnson, N.L., Carini, L.M., Byrnes, E.M., 2013. Multigenerational effects of adolescent morphine exposure on dopamine D2 receptor function. Psychopharmacology 227, 263–272.
Cadet, J.L., Brannock, C., Jayanthi, S., Krasnova, I.N., 2014. Transcriptional and epigenetic substrates of methamphetamine addiction and withdrawal: evidence from a long-access self-administration model in the rat. Mol. Neurobiol. 1–22.
Cadoni, C., Pisanu, A., Solinas, M., Acquas, E., Chiara, G., 2001. Behavioural sensitization after repeated exposure to Δ9-tetrahydrocannabinol and cross-sensitization with morphine. Psychopharmacology 158, 259–266.
Chahrour, M., Jung, S.Y., Shaw, C., Zhou, X., Wong, S.T.C., Qin, J., Zoghbi, H.Y., 2008. MeCP2, a key contributor to neurological disease, activates and represses transcription. Science 320, 1224–1229.
Chase, K.A., Sharma, R.P., 2013. Nicotine induces chromatin remodelling through decreases in the methyltransferases GLP, G9a, Setdb1 and levels of H3K9me2. Int. J. Neuropsychopharmacol. 16, 1129–1138.
Chorbov, V.M., Todorov, A.A., Lynskey, M.T., Cicero, T.J., 2011. Elevated levels of DNA methylation at the OPRM1 promoter in blood and sperm from male opioid addicts. J. Opioid. Manag. 7, 258–264.
Cicero, T.J., Adams, M.L., Giordano, A., Miller, B.T., O'connor, L., Nock, B., 1991. Influence of morphine exposure during adolescence on the sexual maturation of male rats and the development of their offspring. J. Pharmacol. Exp. Ther. 256, 1086–1093.
Cicero, T.J., Adams, M.L., O'connor, L., Nock, B., Meyer, E.R., Wozniak, D., 1990. Influence of chronic alcohol administration on representative indices of puberty and sexual maturation in male rats and the development of their progeny. J. Pharmacol. Exp. Ther. 255, 707–715.
Cone, E.J., Kato, K., Hillsgrove, M., 1996. Cocaine excretion in the semen of drug users. J. Anal. Toxicol. 20, 139–140.
Cuthbertson, K.S., 1983. Parthenogenetic activation of mouse oocytes in vitro with ethanol and benzyl alcohol. J. Exp. Zool. 226, 311–314.
Daxinger, L., Whitelaw, E., 2012. Understanding transgenerational epigenetic inheritance via the gametes in mammals. Nat. Rev. Genet. 13, 153–162.
Demers, C.H., Bogdan, R., Agrawal, A., 2014. The genetics, neurogenetics and pharmacogenetics of addiction. Curr. Behav. Neurosci. Rep. 1, 33–44.
Dick, D.M., Agrawal, A., 2008. The genetics of alcohol and other drug dependence. Alcohol Res. Health 31, 111–118.
Doehring, A., Oertel, B.G., Sittl, R., Lötsch, J., 2013. Chronic opioid use is associated with increased DNA methylation correlating with increased clinical pain. Pain 154, 15–23.
Emanuele, M.A., Wezeman, F., Emanuele, N.V., 2002. Alcohol's effects on female reproductive function. Alcohol Res. Health.
Enoch, M.A., Goldman, D., 1999. Genetics of alcoholism and substance abuse. Psychiatric Clin. N. Am. 22, 289–299.
Everitt, B.J., Parkinson, J.A., Olmstead, M.C., Arroyo, M., Robledo, P., Robbins, T.W., 1999. Associative processes in addiction and reward. The role of amygdala-ventral striatal subsystems. Ann. N. Y. Acad. Sci. 877, 412–438.
Feng, J., Nestler, E.J., 2010. MeCP2 and drug addiction. Nat. Neurosci. 13, 1039–1041.
Finegersh, A., Homanics, G.E., 2014. Paternal alcohol exposure reduces alcohol drinking and increases behavioral sensitivity to alcohol selectively in male offspring. PLoS ONE 9, e99078.
Franklin, T.B., Russig, H., Weiss, I.C., Gräff, J., Linder, N., Michalon, A., Vizi, S., Mansuy, I.M., 2010. Epigenetic transmission of the impact of early stress across generations. Biol. Psychiatry 68, 408–415.
Friedler, G., 1996. Paternal exposures: impact on reproductive and developmental outcome. An overview. Pharmacol. Biochem. Behav. 55, 691–700.
Fürst, Z., Riba, P., Al-Khrasani, M., 2013. New approach to the neurobiological mechanisms of addiction. Neuropsychopharmacol. Hung 15, 189–205.
Gapp, K., Jawaid, A., Sarkies, P., Bohacek, J., Pelczar, P., Prados, J., Farinelli, L., Miska, E., Mansuy, I.M., 2014. Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. Nat. Neurosci. 17, 667–669.
Gatewood, J.M., Cook, G.R., Balhorn, R., Bradbury, E.M., Schmid, C.W., 1987. Sequence-specific packaging of DNA in human sperm chromatin. Science 236, 962–964.
Gaydos, L.J., Wang, W., Strome, S., 2014. Gene repression. H3K27me and PRC2 transmit a memory of repression across generations and during development. Science 345, 1515–1518.
Ghezzi, A., Krishnan, H.R., Lew, L., Prado III, F.J., Ong, D.S., Atkinson, N.S., 2013. Alcohol-induced histone acetylation reveals a gene network involved in alcohol tolerance. PLoS Genet. 9, e1003986.
Goldman, D., Oroszi, G., Ducci, F., 2005. The genetics of addictions: uncovering the genes. Nat. Rev. Genet. 6, 521–532.
Govorko, D., Bekdash, R.A., Zhang, C., Sarkar, D.K., 2012. Male germline transmits fetal alcohol adverse effect on hypothalamic proopiomelanocortin gene across generations. Biol. Psychiatry 72, 378–388.
Hammoud, S., Liu, L., Carrell, D.T., 2009. Protamine ratio and the level of histone retention in sperm selected from a density gradient preparation. Andrologia 41, 88–94.
He, F., Lidow, I.A., Lidow, M.S., 2006. Consequences of paternal cocaine exposure in mice. Neurotoxicol. Teratol. 28, 198–209.
Hillemacher, T., Frieling, H., Moskau, S., Muschler, M.A.N., Semmler, A., Kornhuber, J., Klockgether, T., Bleich, S., Linnebank, M., 2008. Global DNA methylation is influenced by smoking behaviour. Eur. Neuropsychopharmacol. 18, 295–298.
Ho, M.K., Goldman, D., Heinz, A., Kaprio, J., Kreek, M.J., Li, M.D., Munafò, M.R., Tyndale, R.F., 2010. Breaking barriers in the genomics and pharmacogenetics of drug addiction. Clin. Pharmacol. Ther. 88, 779–791.
Host, L., Dietrich, J.-B., Carouge, D., Aunis, D., Zwiller, J., 2011. Cocaine self-administration alters the expression of chromatin-remodelling proteins; modulation by histone deacetylase inhibition. J. Psychopharmacol. Oxf. 25, 222–229.
Hughes, V., 2014. Epigenetics: the sins of the father. Nature 507, 22–24.
Hyman, S.E., Malenka, R.C., Nestler, E.J., 2006. Neural mechanisms of addiction: the role of reward-related learning and memory. Annu. Rev. Neurosci. 29, 565–598.
Im, H.-I., Hollander, J.A., Bali, P., Kenny, P.J., 2010. MeCP2 controls BDNF expression and cocaine intake through homeostatic interactions with microRNA-212. Nat. Neurosci. 13, 1120–1127.
Jamerson, P.A., Wulser, M.J., Kimler, B.F., 2004. Neurobehavioral effects in rat pups whose sires were exposed to alcohol. Brain Res. Dev. Brain Res. 149, 103–111.

Jeanblanc, J., Logrip, M.L., Janak, P.H., Ron, D., 2012. BDNF-mediated regulation of ethanol consumption requires the activation of the MAP kinase pathway and protein synthesis. Eur. J. Neurosci. 37, 607–612.
Kato, Y., Kaneda, M., Hata, K., Kumaki, K., Hisano, M., Kohara, Y., Okano, M., Li, E., Nozaki, M., Sasaki, H., 2007. Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse. Hum. Mol. Genet. 16, 2272–2280.
Kavitharaj, N.K., Vijayammal, P.L., 1998. Nicotine administration induced changes in the gonadal functions in male rats. Pharmacology 58, 2–7.
Kendler, K.S., Prescott, C.A., 1998. Cannabis use, abuse, and dependence in a population-based sample of female twins. Am. J. Psychiatry 155, 1016–1022.
Kendler, K.S., Jacobson, K.C., Prescott, C.A., Neale, M.C., 2003. Specificity of genetic and environmental risk factors for use and abuse/dependence of cannabis, cocaine, hallucinogens, sedatives, stimulants, and opiates in male twins. Am. J. Psychiatry 160, 687–695.
Killinger, C.E., Robinson, S., Stanwood, G.D., 2012. Subtle biobehavioral effects produced by paternal cocaine exposure. Synapse 66, 902–908.
Kim, P., Choi, C.S., Park, J.H., Joo, S.H., Kim, S.Y., Ko, H.M., Kim, K.C., Jeon, S.J., Park, S.H., Han, S.-H., et al., 2014. Chronic exposure to ethanol of male mice before mating produces attention deficit hyperactivity disorder-like phenotype along with epigenetic dysregulation of dopamine transporter expression in mouse offspring. J. Neurosci. Res. 92, 658–670.
Knezovich, J.G., Ramsay, M., 2012. The effect of preconception paternal alcohol exposure on epigenetic remodeling of the h19 and rasgrf1 imprinting control regions in mouse offspring. Epigenomics Epigenetics.
Koob, G.F., Volkow, N.D., 2009. Neurocircuitry of addiction. Neuropsychopharmacology 35, 217–238.
Kreek, M.J., Nielsen, D.A., LaForge, K.S., 2004. Genes associated with addiction. Neuromol. Med. 5, 85–108.
Kumar, A., Choi, K.-H., Renthal, W., Tsankova, N.M., Theobald, D.E.H., Truong, H.-T., Russo, S.J., LaPlant, Q., Sasaki, T.S., Whistler, K.N., et al., 2005. Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. Neuron 48, 303–314.
Kumar, P., Meizel, S., 2005. Nicotinic acetylcholine receptor subunits and associated proteins in human sperm. J. Biol. Chem. 280, 25928–25935.
LaForge, K.S., Yuferov, V., Kreek, M.J., 2000. Opioid receptor and peptide gene polymorphisms: potential implications for addictions. Eur. J. Pharmacol. 410, 249–268.
Lam, M.K.-P., Homewood, J., Taylor, A.J., Mazurski, E.J., 2000. Second generation effects of maternal alcohol consumption during pregnancy in rats. Prog. Neuro-Psychopharmacol. Biological Psychiatry 24, 619–631.
Lane, N., Dean, W., Erhardt, S., Hajkova, P., Surani, A., Walter, J., Reik, W., 2003. Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse. Genesis 35, 88–93.
LaPlant, Q., Vialou, V., Covington, H.E., Dumitriu, D., Feng, J., Warren, B.L., Maze, I., Dietz, D.M., Watts, E.L., Iñiguez, S.D., et al., 2010. Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens. Nat. Neurosci. 13, 1137–1143.
Lee, Y.W., Hennig, B., Fiala, M., Kim, K.S., Toborek, M., 2001. Cocaine activates redox-regulated transcription factors and induces TNF-α expression in human brain endothelial cells. Brain Res. 920, 125–133.
Lerman, C., Berrettini, W., 2003. Elucidating the role of genetic factors in smoking behavior and nicotine dependence. Am. J. Med. Genet. B Neuropsychiatr. Genet. 118B, 48–54.
Levine, A., Huang, Y., Drisaldi, B., Griffin, E.A., Pollak, D.D., Xu, S., Yin, D., Schaffran, C., Kandel, D.B., Kandel, E.R., 2011. Molecular mechanism for a gateway drug: epigenetic changes initiated by nicotine prime gene expression by cocaine. Sci. Transl. Med. 3, 107ra109–107ra109.
Lu, S.C., Mato, J.M., 2005. Role of methionine adenosyltransferase and S-adenosylmethionine in alcohol-associated liver cancer. Alcohol 35, 227–234.
Markou, A., Koob, G.F., 1991. Postcocaine anhedonia. An animal model of cocaine withdrawal. Neuropsychopharmacology 4, 17–26.
Martin, C., Zhang, Y., 2007. Mechanisms of epigenetic inheritance. Curr. Opin. Cell Biol. 19, 266–272.
Maze, I., Nestler, E.J., 2011. The epigenetic landscape of addiction. Ann. N. Y. Acad. Sci. 1216, 99–113.
Maze, I., Covington, H.E., Dietz, D.M., LaPlant, Q., Renthal, W., Russo, S.J., Mechanic, M., Mouzon, E., Neve, R.L., Haggarty, S.J., et al., 2010. Essential role of the histone methyltransferase G9a in cocaine-induced plasticity. Science 327, 213–216.
Maze, I., Feng, J., Wilkinson, M.B., Sun, H., Shen, L., Nestler, E.J., 2011. Cocaine dynamically regulates heterochromatin and repetitive element unsilencing in nucleus accumbens. Proc. Natl. Acad. Sci. U. S. A. 108, 3035–3040.
McGowan, P.O., Meaney, M.J., Szyf, M., 2008. Diet and the epigenetic (re)programming of phenotypic differences in behavior. Brain Res. 1237, 12–24.
Meek, L.R., Myren, K., Sturm, J., Burau, D., 2007. Acute paternal alcohol use affects offspring development and adult behavior. Physiol. Behav. 91, 154–160.
Merikangas, K.R., Stolar, M., Stevens, D.E., Goulet, J., Preisig, M.A., Fenton, B., Zhang, H., O'Malley, S.S., Rounsaville, B.J., 1998. Familial transmission of substance Use disorders. Arch. Gen. Psychiatry 55, 973–979.
Mulé, S.J., Misra, A.L., 1977. Cocaine: Distribution and Metabolism in Animals.
Muthusami, K.R., Chinnaswamy, P., 2005. Effect of chronic alcoholism on male fertility hormones and semen quality. Fertil. Steril. 84, 919–924.
Nielsen, D.A., Utrankar, A., Reyes, J.A., Simons, D.D., Kosten, T.R., 2012. Epigenetics of drug abuse: predisposition or response. Pharmacogenomics 13, 1149–1160.
Nielsen, D.A., Yuferov, V., Hamon, S., Jackson, C., Ho, A., Ott, J., Kreek, M.J., 2008. Increased OPRM1 DNA methylation in lymphocytes of methadone-maintained former heroin addicts. Neuropsychopharmacology 34, 867–873.
Nieratschker, V., Batra, A., Fallgatter, A.J., 2013. Genetics and epigenetics of alcohol dependence. Gene.
Novikova, S.I., He, F., Bai, J., Cutrufello, N.J., Lidow, M.S., 2008. Maternal cocaine administration in mice alters DNA methylation and gene expression in hippocampal neurons of neonatal and prepubertal offspring. PLoS ONE.
Omotoso, G.O., Ibitolu, J.O., Femi-Akinlosotu, O.M., Akinola, O.B., Enaibe, B.U., 2013. Morphological and neurohistological changes in adolescent rats administered with nicotine during intrauterine life. Niger. J. Physiol. Sci. 28, 147–151.
Orozco, L.D., Rubbi, L., Martin, L.J., Fang, F., Hormozdiari, F., Che, N., Smith, A.D., Lusis, A.J., Pellegrini, M., 2014. Intergenerational genomic DNA methylation patterns in mouse hybrid strains. Genome Biol. 15, R68.
Ouko, L.A., Shantikumar, K., Knezovich, J., Haycock, P., Schnugh, D.J., Ramsay, M., 2009. Effect of alcohol consumption on CpG methylation in the differentially methylated regions of H19 and IG-DMR in male Gametes—implications for fetal alcohol spectrum disorders. Alcohol. Clin. Exp. Res. 33, 1615–1627.
Pandey, S.C., Ugale, R., Zhang, H., Tang, L., Prakash, A., 2008. Brain chromatin remodeling: a novel mechanism of alcoholism. J. Neurosci. 28, 3729–3737.
Philibert, R.A., Beach, S.R.H., Gunter, T.D., Brody, G.H., Madan, A., Gerrard, M., 2010. The effect of smoking on MAOA promoter methylation in DNA prepared from lymphoblasts and whole blood. Am. J. Med. Genet. Part B: Neuropsychiatric Genet. 153B, 619–628.
Pistis, M., Perra, S., Pillolla, G., Melis, M., Muntoni, A.L., Gessa, G.L., 2004. Adolescent exposure to cannabinoids induces long-lasting changes in the response to drugs of abuse of rat midbrain dopamine neurons. Bps 56, 86–94.
Ponomarev, I., Wang, S., Zhang, L., Harris, R.A., Mayfield, R.D., 2012. Gene coexpression networks in human brain identify epigenetic modifications in alcohol dependence. J. Neurosci. 32, 1884–1897.
Porcella, A., Gessa, G.L., Pani, L., 1998. Delta9-tetrahydrocannabinol increases sequence-specific AP-1 DNA-binding activity and Fos-related antigens in the rat brain. Eur. J. Neurosci. 10, 1743–1751.
Prak, E.T., Kazazian, H.H., 2000. Mobile elements and the human genome. Nat. Rev. Genet. 1, 134–144.
Pray, L., 2008. Transposons, or jumping genes: not junk DNA. Nat. Educ. 1, 32.
Ramírez, A.R., Castro, M.A., Angulo, C., Ramió, L., Rivera, M.M., Torres, M., Rigau, T., Rodríguez-Gil, J.E., Concha, I.I., 2009. The presence and function of dopamine type 2 receptors in boar sperm: a possible role for dopamine in viability, capacitation, and modulation of sperm motility. Biol. Reprod. 80, 753–761.
Rassoulzadegan, M., Grandjean, V., Gounon, P., Vincent, S., Gillot, I., Cuzin, F., 2006. RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. Nature 441, 469–474.
Ratcliffe, J.M., Gladen, B.C., Wilcox, A.J., Herbst, A.L., 1992. Does early exposure to maternal smoking affect future fertility in adult males? Reprod. Toxicol. 6, 297–307.
Ray, R., Mitra, N., Baldwin, D., Guo, M., Patterson, F., Heitjan, D.F., Jepson, C., Wileyto, E.P., Wei, J., Payne, T., et al., 2010. Convergent evidence that choline acetyltransferase gene variation is associated with prospective smoking cessation and nicotine dependence. Neuropsychopharmacology 35, 1374–1382.
Rice, J.C., Allis, C.D., 2001. Gene regulation: Code of silence. Nature 414, 258–261.
Robison, A.J., Nestler, E.J., 2011. Transcriptional and epigenetic mechanisms of addiction. Nat. Rev. Neurosci. 12, 623–637.
Rodgers, A.B., Morgan, C.P., Bronson, S.L., Revello, S., Bale, T.L., 2013. Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation. J. Neurosci. 33, 9003–9012.
Sachse-Seeboth, C., Pfeil, J., Sehrt, D., Meineke, I., Tzvetkov, M., Bruns, E., Poser, W., Vormfelde, S.V., Brockmöller, J., 2008. Interindividual variation in the pharmacokinetics of Δ9-tetrahydrocannabinol as related to genetic polymorphisms in CYP2C9. Clin. Pharmacol. Ther. 85, 273–276.
Sadri-Vakili, G., Kumaresan, V., Schmidt, H.D., Famous, K.R., Chawla, P., Vassoler, F.M., Overland, R.P., Xia, E., Bass, C.E., Terwilliger, E.F., et al., 2010. Cocaine-induced chromatin remodeling increases brain-derived neurotrophic factor transcription in the rat medial prefrontal cortex, which alters the reinforcing efficacy of cocaine. J. Neurosci. 30, 11735–11744.
Sarkaki, A., Assaei, R., Motamedi, F., Badavi, M., 2008. Effect of parental morphine addiction on hippocampal long-term potentiation in rats offspring. Behav. Brain Res. 186, 72–77.
Sasaki, A., Constantinof, A., Pan, P., 2014. Cocaine exposure prior to pregnancy alters the psychomotor response to cocaine and transcriptional regulation of the dopamine D1 receptor in adult male offspring. Behav. Brain Res. 265, 163–170.
Satta, R., Maloku, E., Zhubi, A., Pibiri, F., Hajos, M., Costa, E., Guidotti, A., 2008. Nicotine decreases DNA methyltransferase 1 expression and glutamic acid decarboxylase 67 promoter methylation in GABAergic interneurons. Proc. Natl. Acad. Sci. U. S. A. 105, 16356–16361.
Schuel, H., Burkman, L.J., Lippes, J., Crickard, K., Forester, E., Piomelli, D., Giuffrida, A., 2002. N-Acylethanolamines in human reproductive fluids. Chem. Phys. Lipids 121, 211–227.
Sheng, J., Lv, Z.G., Wang, L., Zhou, Y., Hui, B., 2011. Histone H3 phosphoacetylation is critical for heroin-induced place preference. NeuroReport 22, 575.
Singer, T., McConnell, M.J., Marchetto, M.C.N., Coufal, N.G., Gage, F.H., 2010. LINE-1 retrotransposons: mediators of somatic variation in neuronal genomes? Trends Neurosci. 33, 345–354.

Skinner, M.K., 2008. What is an epigenetic transgenerational phenotype? Reprod. Toxicol. 25, 2–6.
Skinner, M.K., 2011. Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability. Epigenetics 6, 838–842.
Slotkin, R.K., Martienssen, R., 2007. Transposable elements and the epigenetic regulation of the genome. Nat. Rev. Genet. 8, 272–285.
Steketee, J.D., Kalivas, P.W., 2011. Drug Wanting: behavioral sensitization and relapse to drug-seeking behavior. Pharmacol. Rev. 63, 348–365.
Stouder, C., Somm, E., Paoloni-Giacobino, A., 2011. Prenatal exposure to ethanol: a specific effect on the H19 gene in sperm. Reprod. Toxicol. 31, 507–512.
Sun, H., Maze, I., Dietz, D.M., Scobie, K.N., Kennedy, P.J., Damez-Werno, D., Neve, R.L., Zachariou, V., Shen, L., Nestler, E.J., 2012. Morphine epigenomically regulates behavior through alterations in histone H3 lysine 9 dimethylation in the nucleus accumbens. J. Neurosci. 32, 17454–17464.
Sweet, F., Murdock, G.L., 1987. Affinity labeling of hormone-specific proteins. Endocr. Rev. 8, 154–184.
Szutorisz, H., DiNieri, J.A., Sweet, E., Egervari, G., Michaelides, M., Carter, J.M., Ren, Y., Miller, M.L., Blitzer, R.D., Hurd, Y.L., 2014. Parental THC exposure leads to compulsive heroin-seeking and altered striatal synaptic plasticity in the subsequent generation. Neuropsychopharmacology 39, 1315–1323.
Toledo Rodriguez, M., Lotfipour, S., Leonard, G., Perron, M., Richer, L., Veillette, S., Pausova, Z., Paus, T., 2010. Maternal smoking during pregnancy is associated with epigenetic modifications of the brain-derived neurotrophic factor-6 exon in adolescent offspring. Am. J. Med. Genet. Part B: Neuropsychiatric Genet. 153B, 1350–1354.
Trivedi, M., Shah, J., Hodgson, N., Byun, H.-M., Deth, R., 2014. Morphine induces redox-based changes in global DNA methylation and retrotransposon transcription by inhibition of excitatory amino acid transporter type 3-mediated cysteine uptake. Mol. Pharmacol. 85, 747–757.
Tsuang, M.T., Lyons, M.J., Eisen, S.A., Goldberg, J., True, W., Lin, N., Meyer, J.M., Toomey, R., Faraone, S.V., Eaves, L., 1996. Genetic influences on DSM-III-R drug abuse and dependence: a study of 3,372 twin pairs. Am. J. Med. Genet. 67, 473–477.
Tsuang, M.T., Bar, J.L., Harley, R.M., Lyons, M.J., 2001. The Harvard twin study of substance abuse: what we have learned. Harv. Rev. Psychiatry 9, 267–279.
Tsuang, M.T., Lyons, M.J., Meyer, J.M., Doyle, T., Eisen, S.A., Goldberg, J., True, W., Lin, N., Toomey, R., Eaves, L., 1998. Co-occurrence of abuse of different drugs in Men: the role of drug-specific and Shared Vulnerabilities. Arch. Gen. Psychiatry 55, 967–972.
van der Knaap, L.J., Schaefer, J.M., Franken, I.H.A., Verhulst, F.C., van Oort, F.V.A., Riese, H., 2014. Catechol-O-methyltransferase gene methylation and substance use in adolescents: the TRAILS study. Genes. Brain Behav. (n/a–n/a).
Vassoler, F.M., Byrnes, E.M., Pierce, R.C., 2014. The impact of exposure to addictive drugs on future generations: physiological and behavioral effects. Neuropharmacology 76, 269–275.
Vassoler, F.M., Johnson, N.L., Byrnes, E.M., 2013a. Female adolescent exposure to cannabinoids causes transgenerational effects on morphine sensitization in female offspring in the absence of in utero exposure. J. Psychopharmacol. Oxf. 27, 1015–1022.
Vassoler, F.M., White, S.L., Schmidt, H.D., Sadri-Vakili, G., Pierce, R.C., 2013b. Epigenetic inheritance of a cocaine-resistance phenotype. Nat. Neurosci. 16, 42–47.
Vermeulen-Smit, E., Koning, I.M., Verdurmen, J.E.E., Van der Vorst, H., Engels, R.C.M.E., Vollebergh, W.A.M., 2012. The influence of paternal and maternal drinking patterns within two-partner families on the initiation and development of adolescent drinking. Addict. Behav. 37, 1248–1256.
Verweij, K.J.H., Zietsch, B.P., Lynskey, M.T., Medland, S.E., Neale, M.C., Martin, N.G., Boomsma, D.I., Vink, J.M., 2010. Genetic and environmental influences on cannabis use initiation and problematic use: a meta-analysis of twin studies. Addiction 105, 417–430.
Vink, J.M., Willemsen, G., Boomsma, D.I., 2005. Heritability of smoking initiation and nicotine dependence. Behav. Genet. 35, 397–406.
Volkow, N.D., Wang, G.-J., Fowler, J.S., Logan, J., Gatley, S.J., Gifford, A., Hitzemann, R., Ding, Y.-S., Pappas, N., 1999. Prediction of reinforcing responses to psychostimulants in humans by brain dopamine D2 receptor levels. Am. J. Psychiatry 156, 1440–1443.
Volkow, N.D., Wang, G.-J., Fowler, J.S., Tomasi, D., Telang, F., Baler, R., 2010. Addiction: decreased reward sensitivity and increased expectation sensitivity conspire to overwhelm the brain's control circuit. Bioessays 32, 748–755.
Vyssotski, D.L., 2011. Transgenerational epigenetic compensation. Evolocus.
Wang, X., Gao, W., Ma, X., Wang, X., Song, C., Huang, X., Liu, H., 2014a. Dot1L mediated histone H3 lysine79 methylation is essential to meiosis progression in mouse oocytes. Neuro Endocrinol. Lett. 35, 523–530.
Wang, Y., Lai, J., Cui, H., Zhu, Y., Zhao, Bin, Wang, W., Wei, S., 2014b. Inhibition of histone deacetylase in the basolateral amygdala facilitates morphine context-associated memory formation in rats. J. Mol. Neurosci. 1–10.
Whitelaw, E., Morgan, H.D., Sutherland, H.G.E., Martin, D.I.K., 1999. Epigenetic inheritance at the agouti locus in the mouse. Nat. Genet. 23, 314–318.
Wise, R.A., 1998. Wise: Drug-activation of Brain Reward Pathways – Google Scholar (Drug and alcohol dependence).
Wozniak, D.F., Cicero, T.J., Kettinger III, L., Meyer, E.R., 1991. Paternal alcohol consumption in the rat impairs spatial learning performance in male offspring. Psychopharmacology 105, 289–302.
Wykes, S.M., Krawetz, S.A., 2003. The structural organization of sperm chromatin. J. Biol. Chem. 278, 29471–29477.
Xu, M., Hu, X.-T., Cooper, D.C., Moratalla, R., Graybiel, A.M., White, F.J., Tonegawa, S., 1994. Elimination of cocaine-induced hyperactivity and dopamine-mediated neurophysiological effects in dopamine D1 receptor mutant mice. Cell 79, 945–955.
Yang, X., Hegde, V.L., Rao, R., Zhang, J., Nagarkatti, P.S., Nagarkatti, M., 2014. Histone modifications are associated with Δ9-tetrahydrocannabinol-mediated alterations in antigen-specific T cell responses. J. Biol. Chem. 289, 18707–18718.
Yazigi, R.A., Odem, R.R., Polakoski, K.L., 1991. Demonstration of specific binding of cocaine to human spermatozoa. Jama 266, 1956–1959.
Yochum, C., Doherty-Lyon, S., Hoffman, C., Hossain, M.M., Zelikoff, J.T., Richardson, J.R., 2014. Prenatal cigarette smoke exposure causes hyperactivity and aggressive behavior: role of altered catecholamines and BDNF. Exp. Neurol. 254, 145–152.
Zajac, C., Ivery, E., Abel, E., 1989. Hippocampal morphology in paternally alcohol-exposed offspring of Long-Evans rats. Alcohol. Clin. Exp. Res. 13, 383.
Zhu, J., Lee, K.P., Spencer, T.J., Biederman, J., Bhide, P.G., 2014. Transgenerational transmission of hyperactivity in a mouse model of ADHD. J. Neurosci. 34, 2768–2773.
Zhu, J., Zhang, X., Xu, Y., Spencer, T.J., Biederman, J., Bhide, P.G., 2012. Prenatal nicotine exposure mouse model showing hyperactivity, reduced cingulate cortex volume, reduced dopamine turnover, and responsiveness to oral methylphenidate treatment. J. Neurosci. 32, 9410–9418.

Rehan et al. BMC Medicine 2012, 10:129
http://www.biomedcentral.com/1741-7015/10/129

BMC Medicine

RESEARCH ARTICLE Open Access

Perinatal nicotine exposure induces asthma in second generation offspring

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Abstract

Background: By altering specific developmental signaling pathways that are necessary for fetal lung development, perinatal nicotine exposure affects lung growth and differentiation, resulting in the offsprings' predisposition to childhood asthma; peroxisome proliferator-activated receptor gamma (PPARγ) agonists can inhibit this effect. However, whether the perinatal nicotine-induced asthma risk is restricted to nicotine-exposed offspring only; whether it can be transmitted to the next generation; and whether PPARγ agonists would have any effect on this process are not known.

Methods: Time-mated Sprague Dawley rat dams received either placebo or nicotine (1 mg/kg, s.c.), once daily from day 6 of gestation to postnatal day (PND) 21. Following delivery, at PND21, generation 1 (F1) pups were either subjected to pulmonary function tests, or killed to obtain their lungs, tracheas, and gonads to determine the relevant protein markers (mesenchymal contractile proteins), global DNA methylation, histone 3 and 4 acetylation, and for tracheal tension studies. Some F1 animals were used as breeders to generate F2 pups, but without any exposure to nicotine in the F1 pregnancy. At PND21, F2 pups underwent studies similar to those performed on F1 pups.

Results: Consistent with the asthma phenotype, nicotine affected lung function in both male and female F1 and F2 offspring (maximal 250% increase in total respiratory system resistance, and 84% maximal decrease in dynamic compliance following methacholine challenge; $P < 0.01$, nicotine versus control; $P < 0.05$, males versus females; and $P > 0.05$, F1 versus F2), but only affected tracheal constriction in males (51% maximal increase in tracheal constriction following acetylcholine challenge, $P < 0.01$, nicotine versus control; $P < 0.0001$, males versus females; $P > 0.05$, F1 versus F2); nicotine also increased the contractile protein content of whole lung (180% increase in fibronectin protein levels, $P < 0.01$, nicotine versus control, and $P < 0.05$, males versus females) and isolated lung fibroblasts (for example, 45% increase in fibronectin protein levels, $P < 0.05$, nicotine versus control), along with decreased PPARγ expression (30% decrease, $P < 0.05$, nicotine versus control), but only affected contractile proteins in the male trachea ($P < 0.05$, nicotine versus control, and $P < 0.0001$, males versus females). All of the nicotine-induced changes in the lung and gonad DNA methylation and histone 3 and 4 acetylation were normalized by the PPARγ agonist rosiglitazone except for the histone 4 acetylation in the lung.

Conclusions: Germline epigenetic marks imposed by exposure to nicotine during pregnancy can become permanently programmed and transferred through the germline to subsequent generations, a ground-breaking finding that shifts the current asthma paradigm, opening up many new avenues to explore.

Keywords: nicotine, lung, epigenetic, asthma, multigenerational, gender difference

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Background

Asthma is a major public health problem [1,2]. It is the most common chronic disease of childhood [3,4], resulting in a significant medical burden and the resultant healthcare costs [5,6]. The burden of this disease is increasing rapidly, with the most striking increases seen among children, the prevalence rates in some populations being greater than 30% [7]. Although a multitude of causes contribute to childhood asthma, maternal smoking during pregnancy is a well-established contributor [8-13], and it is a major modifiable risk factor, the elimination of which could significantly reduce the prevalence of childhood asthma. However, given that the tobacco industry continues to spend billions of dollars on advertising to attract young smokers, it is unlikely that the problem of smoking during pregnancy will go away any time soon [14]. Approximately 250,000,000 women smoke daily world-wide. Twelve percent of US women still continue to smoke during pregnancy, resulting in the births of at least 400,000 smoke-exposed infants per year in the US [15,16]. This aspect of the smoking-induced asthma etiology is particularly important since there is emerging evidence that, following *in utero* exposure to maternal smoke, asthma can be transmitted multigenerationally. For example, a questionnaire in the Children's Health Study from Southern California reported that grandmaternal smoking during pregnancy increases the risk of asthma in grandchildren regardless of the presence of maternal smoking [17]. Yet there is neither experimental evidence nor any mechanistic explanation for this phenomenon. Using a well-established rat model of *in utero* nicotine exposure for childhood asthma [18-20], we aimed to determine if *in utero* nicotine exposure would transmit asthma to the second generation offspring, and if epigenetic mechanism(s) could be involved in this transmission.

The mechanism linking smoke/nicotine exposure during pregnancy to multigenerational (MG) transmission of asthma is unknown. Since transmission via the germline is the most likely explanation for the non-genetic MG transmission phenomenon, we rationalized that epigenetic modifications of the germline explain MG transmission of perinatal nicotine-induced asthma. For example, a change in the organism's environment, that is, smoke/nicotine exposure (of the F0 generation), can result in modifications in gene expression through alterations in DNA methylation, histone (H) modification, non-coding RNA, and/or protein structure and assembly without changing the DNA sequence of the F1 germline, which can be transmitted to subsequent generations [21,22]. However, whether epigenetic marks such as alterations in DNA methylation and/or H modifications acquired in one generation can be inherited by the next generation is not clear. While one study reported that the acquired DNA methylation marks are not transmitted to subsequent generations [23], another study came to the opposite conclusion [24]. We hypothesize that smoke/nicotine-induced epigenetic marks can become permanently programmed and transferred through the germline to subsequent generations, resulting in altered phenotypic changes at the cellular and consequent organismal levels (for example, asthma in the case of *in utero* smoke/nicotine exposure) in the offspring over multiple subsequent generations, for example, F2, F3, and so on.

Furthermore, the effect of the child's gender on asthma risk following perinatal exposure to cigarette smoke is not clear since an increased risk in both boys [25] and girls [26] has previously been reported. However, since there is emerging evidence to suggest that finely-tuned developmental programs, such as that of the lung, may be sensitive to specific environmental challenges in a sex-specific manner, particularly during the developmental programming and gametogenesis stages [27], and since maternal smoking is known to affect fetal growth more profoundly in the male fetus [28], we also hypothesized that there would be a sexual dimorphism in asthma risk following *in utero* smoke exposure, with males being more susceptible than females.

Methods

Materials

Nicotine bitartrate was acquired from Sigma-Aldrich (St. Louis, MO, USA) and rosiglitazone (RGZ) from Cayman Chemical (Chicago, IL, USA). DNA methylation (cat#: P-1034-96), H3 (cat#: P-4008-96) and H4 (cat#: P-4009-96) acetylation kits were obtained from Epigentek (Farmingdale, NY, USA). All plasticware and culture media were purchased from Corning (Corning, NY, USA) and Invitrogen, Inc. (San Deigo, CA, USA).

Animal model (Schematic 1)

Pathogen-free timed (embryonic day 0 = day of mating) pregnant Sprague-Dawley F0 rats (200 to 250 g body weight) were obtained from Charles River (Hollister, CA, USA), and allowed to acclimatize until embryonic day 6 [see Additional File 1 for Schematic]. Dams were randomized to receive placebo (diluent, normal saline), nicotine (1 mg/kg, s.c.) alone, or nicotine (1 mg/kg), and the PPARγ agonist RGZ (3 mg/kg, i.p.) in 100 μL volumes once daily from embryonic day 6 to postnatal day (PND) 21. The dams were allowed free access to water and pair-fed according to the previous day's intake by the nicotine group animals and were maintained in a 12:12-hour light-dark cycle. Following delivery at term, the F1 pups were allowed to breast feed *ad libitum*. At PND21 the pups were either subjected to pulmonary function tests

(PFTs) and then killed, or killed and their lungs and tracheas collected for airway contractility protein levels, fibroblast isolation, and tracheal tension studies. The gonads were collected to determine epigenetic marks. Some F1 animals were weaned at PND21 and maintained as breeders to generate F2 rats, but without any exposure to nicotine in the F1 pregnancy. At PND21, F2 animals underwent studies similar to those performed on F1 animals. All studies were approved by the Los Angeles Biomedical Research Institutional Review Board and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The dose (1 mg/kg) chosen for the nicotine treatment has previously been shown to result in an asthma phenotype in the offspring in a number of studies (reviewed in [18]) and is comparable to the dose of nicotine to which habitual smokers are exposed, that is, approximately 1 mg/kg/body weight [29]. As further support for the doses of nicotine and RGZ used in this study, pulmonary function changes induced following perinatal exposure to 1 mg/kg/day nicotine, administered s.c., were recently shown to be blocked by the concomitant i.p. administration of 3 mg/kg/day RGZ (20). It is also important to note that this dose of RGZ has previously been shown to block hyperoxia-induced neonatal lung injury as well [30]. Two animals were used for each condition per experiment, and each experiment was repeated at least three times.

Lung fibroblast isolation

PND21 rat lung fibroblasts were cultured according to previously described methods, with slight modifications [30]. Briefly, the lungs were removed and put into Hanks' balanced salt solution (HBSS) and chopped into small pieces. The HBSS was decanted and 5 volumes of 0.05% trypsin were added to the lung preparation. The lungs were dissociated in a 37°C water bath using a Teflon stirring bar to disrupt the tissue mechanically. Once the tissue was dispersed into a unicellular suspension, the cells were pelleted at 500 × *g* for 10 minutes at room temperature in a 50-mL polystyrene centrifuge tube. The supernatant was decanted, and the pellet was resuspended in minimal essential medium (MEM) containing 10% FBS to yield a mixed cell suspension of approximately 3×10^8 cells, as determined using a Coulter particle counter (Beckman-Coulter, Hayaleah, FL, USA). The cell suspension was then added to culture flasks (75 cm^2) for 30 to 60 minutes to allow for the differential adherence of lung fibroblasts. These cells are greater than 95% pure fibroblasts based upon vimentin-positive staining.

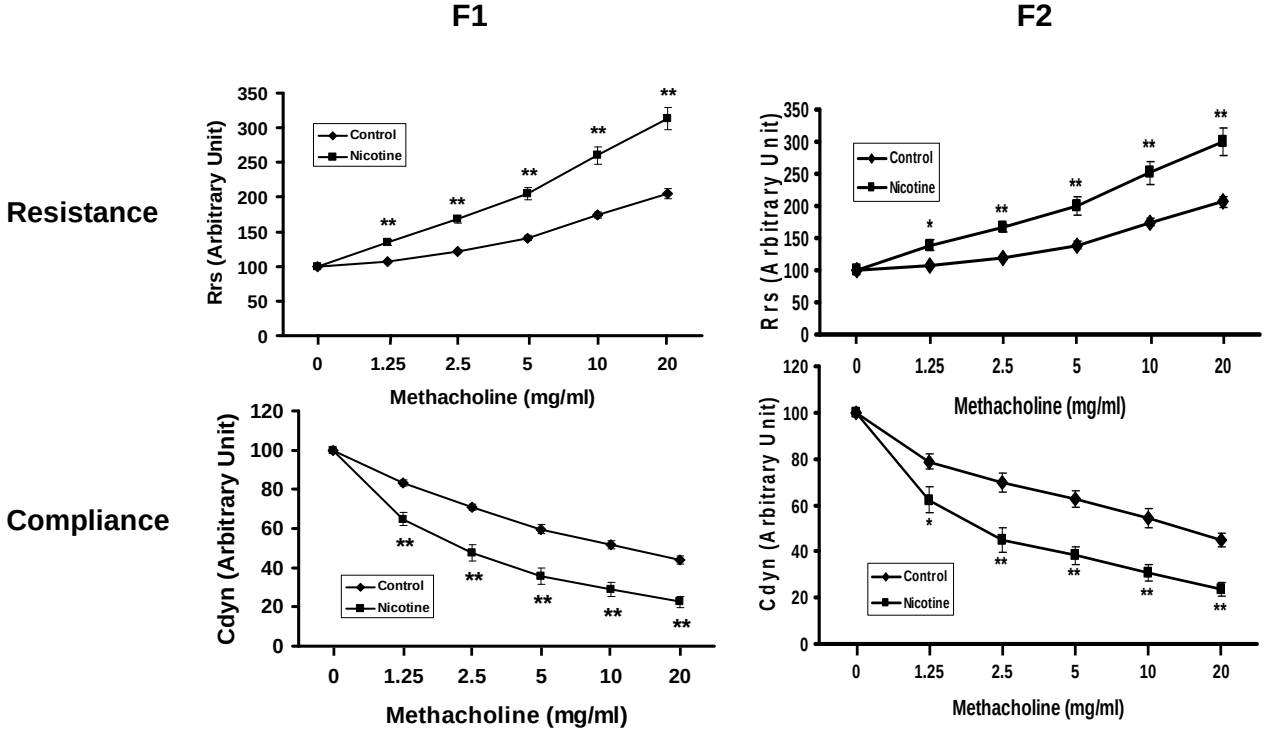

Figure 1 Effect of perinatal nicotine exposure on mixed gender offspring pulmonary function. Compared to the control group, with nicotine administration there was a significant increase in total airway resistance and a decrease in total compliance following Mch challenge in both F1 and F2 rats of mixed gender even though the F2 rats were not exposed to any nicotine during the F1 gestation. Values are means ± SE. n = 10 to 12 for each group. *$P < 0.05$, **$P < 0.01$, versus control. Mch, methacholine.

Pulmonary function testing

Measurement of respiratory function was performed with a plethysmograph for restrained animals (Buxco Inc, Troy, NY, USA); the pups were deeply anesthetized and sedated with ketamine (70 mg/kg, Bioniche Teoranta Inverin, Co., Galway, Ireland), and xylazine (7 mg/kg, Akorn, Inc., Decatur, IL, USA), tracheostomized and ventilated. Rats were exposed to increasing concentrations of aerosolized methacholine (0, 1.25, 2.5, 5, 10, and 20 mg/ml) over a period of 3 minutes. Lung resistance (Rrs) and dynamic compliance (Cdyn) were plotted as a function of the methacholine concentration administered.

Tracheal constriction studies

The trachea was excised *en bloc* immediately after sacrifice and dissected free of connective tissue in ice-cold modified Krebs-Ringer bicarbonate buffer (in mM: 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$ and 11.1 glucose). Subsequently, an approximately 6 mm tracheal ring was resected from the midsection and used for tracheal tension studies. The tracheal ring was suspended in an organ chamber filled with 10 ml of modified Krebs-Ringer bicarbonate solution maintained at 37 ± 0.5° C and aerated with 95% O_2-5% CO_2 (pH 7.4). Each ring was suspended via two stirrups that were passed through the lumen: one stirrup was anchored to the bottom of the organ chamber and the other stirrup was connected to a strain gauge (model FT03C, Grass Instrument, Quincy, MA, USA) for the measurement of isometric force, as described previously [20].

At the beginning of the experiment, each tracheal ring was stretched to its optimal resting tension, which was achieved by step-wise stretching in 0.1-g increments until the contractile response to 100 mM KCl reached a plateau. The optimal resting tension was measured, and then each tracheal ring was allowed to equilibrate for one hour after it was brought to its optimal resting tension. The effects of acetylcholine were determined at least 30 minutes after the administration of nitro-L-arginine (1×10^{-4} M, an inhibitor of nitric oxide synthase). In all experiments, indomethacin (1×10^{-5} M) was added to the bath to prevent the possible interference by prostanoids.

Western blot

Western blot analysis for fibronectin, α-smooth muscle actin (α-SMA), calponin, collagens I and III, nicotinic acetylcholine receptors α3 and α7, and PPARγ were performed as described previously [20].

Real Time Reverse Transcription-Polymerase Chain Reaction

RT-PCR was performed as previously described [19,31].

Global DNA methylation

Genomic DNA from lungs, testes, and ovaries was isolated using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA, Cat. No.: 51304) and the DNA concentration was determined using a NanoDrop2000. Global DNA methylation was quantified using a MethylFlash Methylated DNA Quantification Kit (Epigentek, Cat. No.: P-1034) according to the manufacturer's instructions. Briefly, DNA is bound to strip wells that are specifically treated to have high DNA affinity. The methylated fraction of DNA is detected using capture and detection antibodies, and then quantified colorimetrically by reading the absorbance at 450 nm in a Wallac 1420 Multilabel Counter. The amount and

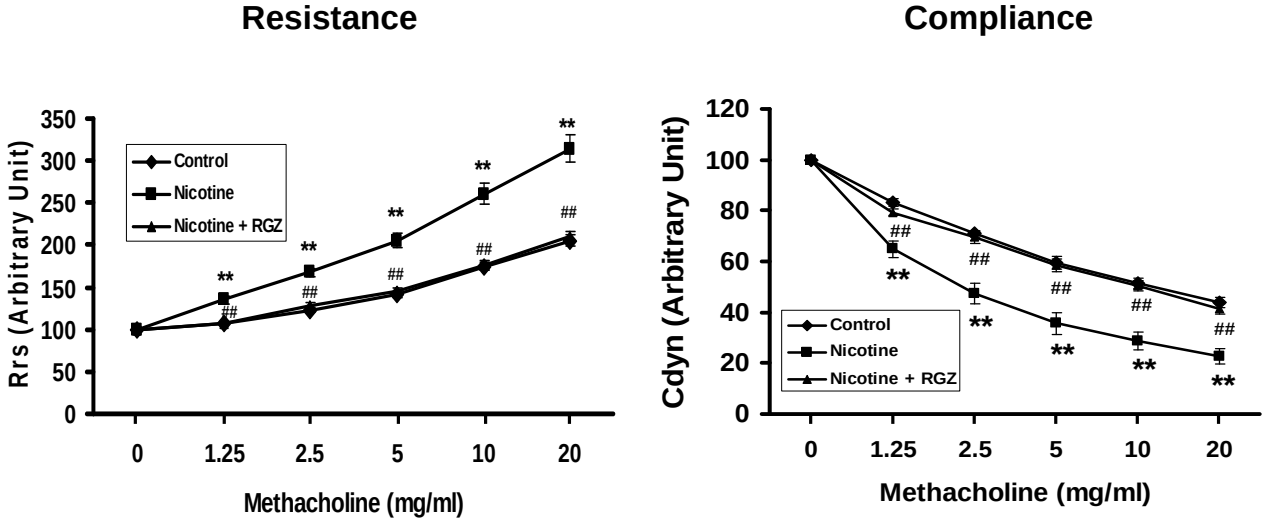

Figure 2 Effect of rosiglitazone (RGZ) on perinatal nicotine exposure-induced alterations in mixed gender offspring pulmonary function. Compared to the control group, with nicotine administration there was a significant increase in resistance and a significant decrease in compliance of the lung following a Mch challenge, both of which were blocked by concomitant RGZ administration. Values are means ± SE. n = 10 to 12 for each group. **P < 0.01, versus control; ##P < 0.01, versus nicotine group. Mch, methacholine.

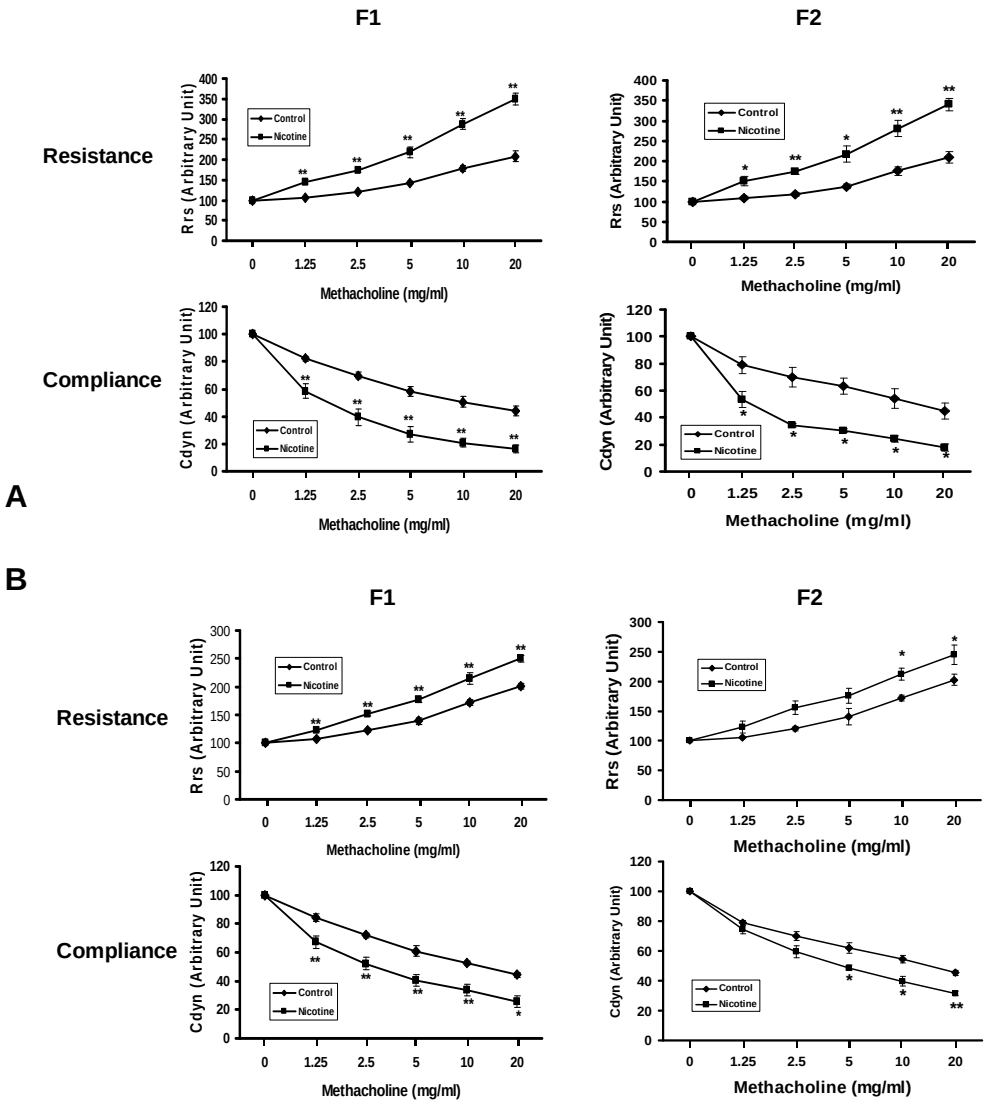

Figure 3 Effect of perinatal nicotine exposure on male and female offspring pulmonary function. Compared to the control group, with nicotine administration there were significant increases in resistance and decreases in compliance of the lung following Mch challenge in F1 and F2 male (**A**) and female (**B**) rats even though the F2 pups were not exposed to any nicotine during gestation. Values are means ± SE. n = 5 to 6 for each group. *$P < 0.05$, **$P < 0.01$ versus control. Mch, methacholine.

percentage of methylated DNA (5-mC) in the total DNA extract was calculated based on a standard curve generated using a methylated DNA positive control.

Global histone H3 and H4 acetylation

Histone protein from lungs, testes, and ovaries was extracted using an EpiQuik Total Histone Extraction Kit (Epigentek, Cat. No.: OP-0006), and histone protein concentration was measured by the Bradford method, using BSA as the standard. Global histone H3 and H4 acetylation was quantified using an EpiQuik Global Histone H3 Acetylation Assay Kit (Epigentek, Cat. No.: P-4008) and an EpiQuik Global Histone H4 Acetylation Assay Kit (Epigentek, Cat. No.: P-4009) according to the manufacturer's instructions. Briefly, the histone proteins are stably spotted on the strip wells. The acetylated histone H3 and H4 are recognized with high-affinity antibodies against them. The amount of acetylated H3 and H4 are quantified through a horseradish peroxidase-conjugated secondary antibody-color development system, and the absorbance at 450 nm is read in a Wallac 1420 Multilabel Counter. The amount of acetylated histone H3 and H4 in the total histone protein was calculated according to a standard curve generated using acetylated histone H3 or H4.

Statistics

The data for analysis were obtained from at least three independent sets of experiments. Analysis of variance (ANOVA) for multiple comparisons with Bonferroni *post-hoc* analysis and Student's t-test, as indicated, were used, and $P < 0.05$ was considered to indicate significant differences among the experimental groups.

Results

We initially determined the effect of nicotine on pulmonary function in response to a graded methacholine challenge. Compared to the control group, with perinatal nicotine exposure to F0 dams (Figure 1) there was a significant increase in total airway Rrs, and a decrease in total airway Cdyn of the respiratory system, not only in F1 rats [20], but also in F2 rats ($P < 0.01$ versus control for both Rrs and Cdyn), even though the F2 rats were not exposed to nicotine. In accord with our recently published data on F1 rats [20], RGZ treatment blocked the nicotine-induced increase in Rrs and decrease in Cdyn in F2 rats ($P < 0.01$ versus nicotine for both Rrs and Cdyn) (Figure 2).

We subsequently analyzed the effects of nicotine exposure on PFT's in male and female offspring separately (Figure 3a and 3b). Following a methacholine challenge, perinatal nicotine exposure only to F0 dams caused a significant increase in Rrs and a decrease in Cdyn in both the males and females of the F1 and F2 generations ($P < 0.05$, males versus females, for both Rrs and Cdyn).

Having determined the effect of *in utero* nicotine exposure on the F1 and F2 offspring whole respiratory systems, we examined the effect of nicotine on tracheal rings isolated from F1 and F2 offspring (Figure 4). Nicotine administration caused a significant increase in tracheal constriction in response to acetylcholine only in the males of both the F1 and F2 generations ($P < 0.01$ versus control).

Since we had previously determined that the nicotine exposure of F0 dams increased the expression of lung contractile proteins and inhibited PPARγ expression in both the whole lung and isolated alveolar interstitial fibroblasts of F1 rats [20], we next determined this effect in the F2 offspring. We found that the protein levels of fibronectin, α-SMA, calponin, and collagens I and III, and nicotinic acetylcholine receptors α3 and α7 were all increased significantly in the whole lung lysates of both the male (Figure 5a and 5b) and female F2 ($P < 0.05$ versus control for all) (Figure 6a and 6b) rats. In contrast, at the tracheal

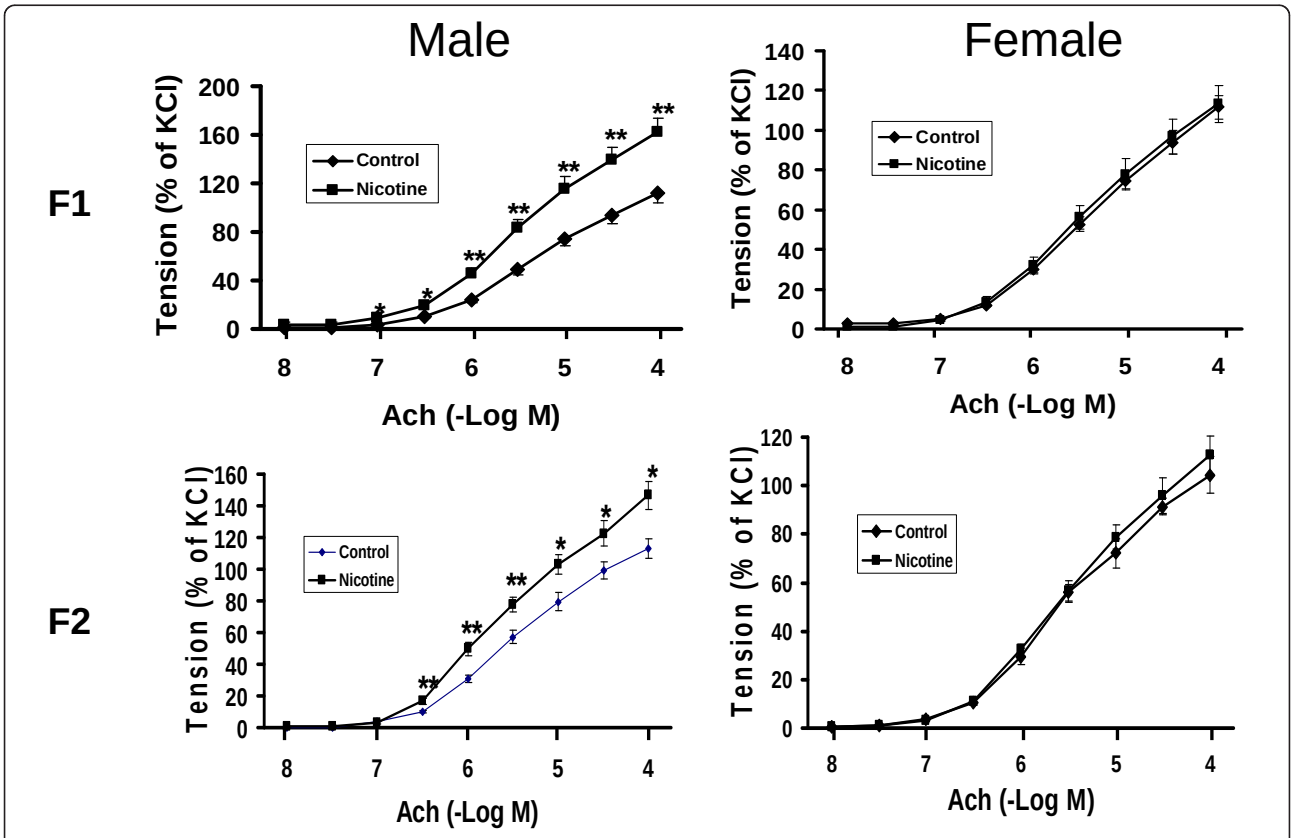

Figure 4 Effect of perinatal nicotine exposure on acetylcholine tracheal constriction response in F1 and F2 offspring. Compared to the control group, with nicotine administration there was a significant increase in tracheal constriction in response to acetylcholine only in the males of both the F1 and F2 generations. Values are means ± SE; * $P < 0.05$, ** $P < 0.01$, versus control group; n = 5 to 6.

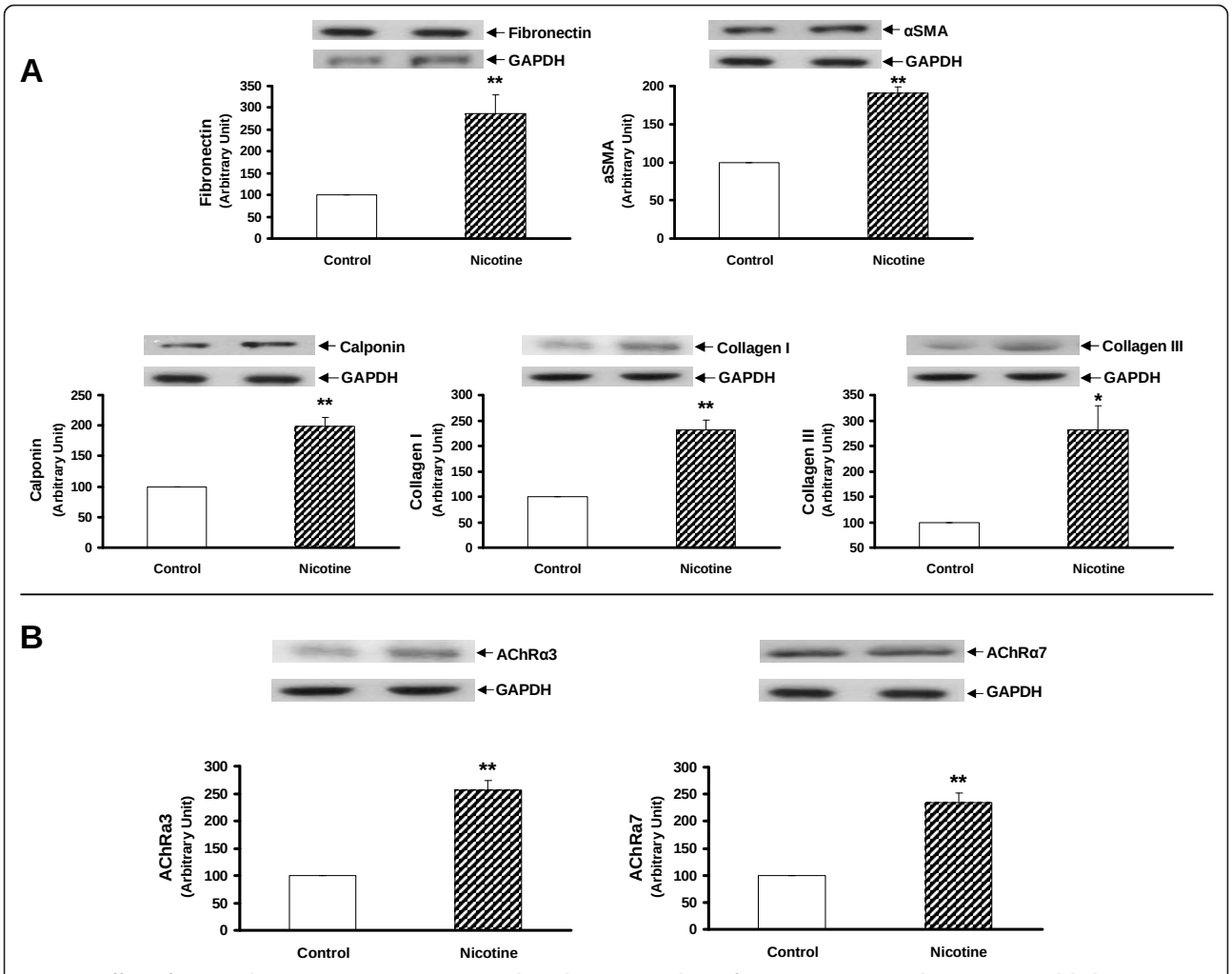

Figure 5 Effect of perinatal nicotine exposure on mesenchymal protein markers of airway reactivity and nicotinic acetylcholine receptors α3 and α7 in lungs of male F2 rats. Compared to the control group, with nicotine administration the protein levels of fibronectin, α-SMA, calponin, and collagens I and III (**A**) and nicotinic acetylcholine receptors α3 and α7 (**B**) increased significantly. Upper panels show representative Western blots for these markers and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Lower panels show the densitometric values of the markers normalized to GAPDH. Values are means ± SE. n = 6 for each group. * $P < 0.05$, ** $P < 0.01$, versus control group. α-SMA, α-smooth muscle actin.

level the expression of fibronectin, α-SMA, calponin, collagen I, nicotinic acetylcholine receptors (nAChR) α3 and α7 were increased only in males ($P < 0.05$ versus control) (Figure 7a and 7b). In line with our recently published data, the expression of fibronectin was increased, and that of PPARγ was decreased, at both the mRNA and protein levels in the alveolar interstitial fibroblasts isolated from the lungs of F2 rats ($P < 0.05$ versus control) (Figure 8).

To determine the likely mechanism of nicotine-induced transmission of asthma to the second generation offspring, we next determined the effect of F0 nicotine exposure on global DNA methylation and histone acetylation in the lungs and gonads of the F1 rats. We observed that with nicotine administration global DNA methylation increased in the testes ($P < 0.01$), decreased in the ovaries ($p < 0.05$), but did not change in the lungs (Figure 9a); H3 acetylation increased in the lungs ($P < 0.01$) and testes ($P < 0.05$), but did not change in the ovaries (Figure 9b); H4 acetylation decreased in the lungs ($P < 0.01$) while it increased in the testes ($P < 0.01$) and ovaries ($P < 0.05$) (Figure 9c). Further, we determined if the nicotine effects on histone methylation and acetylation were affected by RGZ, which has been shown to normalize the structural and functional effects of nicotine on the offspring lung phenotype at F1 [20]. The nicotine-induced increase in lung H3 acetylation was blocked (Figure 9b) by RGZ treatment, whereas there was no effect on global DNA methylation (Figure 9a) or on the nicotine-induced decrease in H4 acetylation (Figure 9c), suggesting a central role of H3 acetylation in mediating nicotine's effects on the pulmonary phenotype. Finally, it is reassuring to note that perinatal exposure of

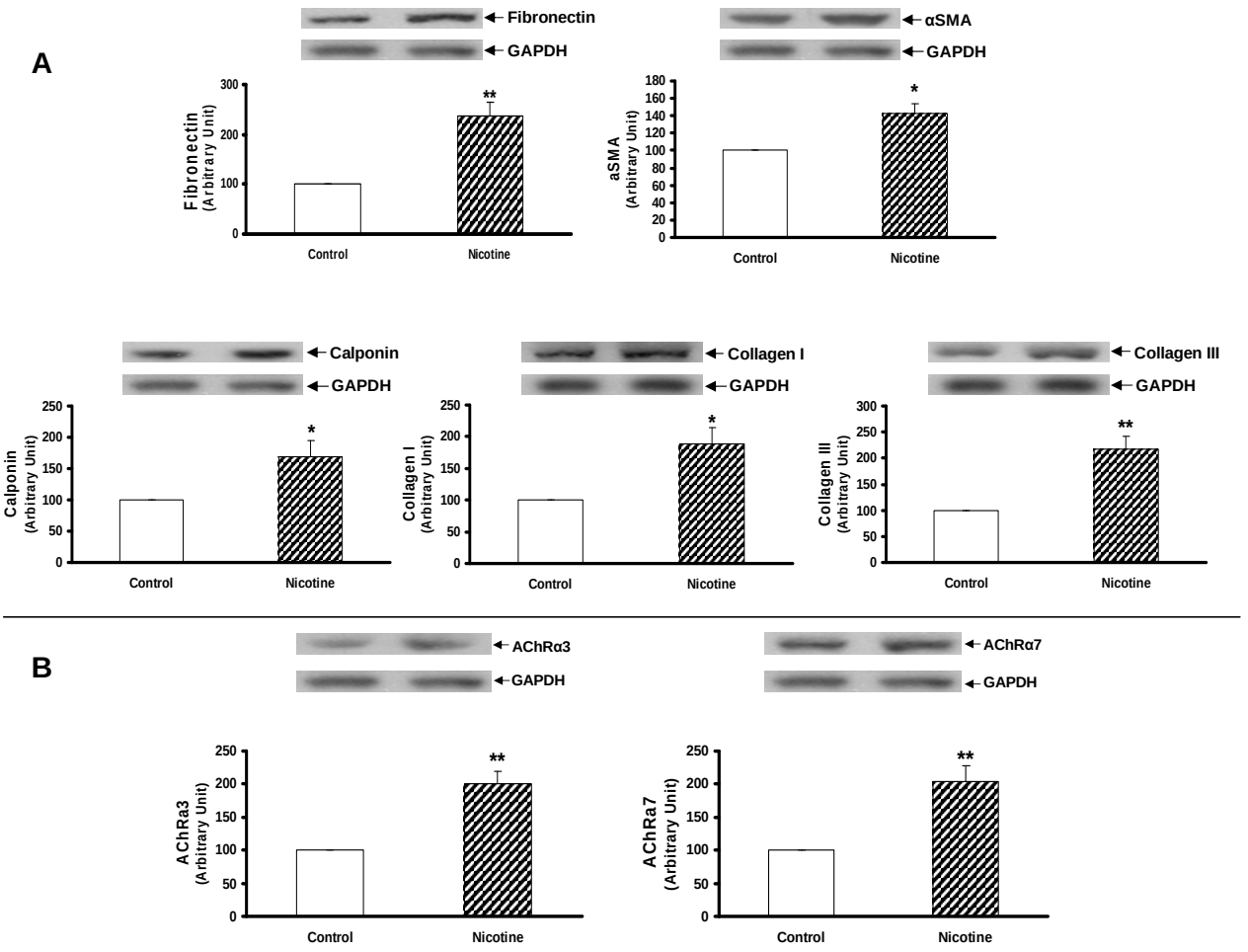

Figure 6 Effect of perinatal nicotine exposure on mesenchymal protein markers of airway reactivity and nicotinic acetylcholine receptors α3 and α7 in lungs of female F2 rats. Compared to the control group, with nicotine administration the protein levels of fibronectin, α-SMA, calponin, and collagens I and III (**A**) and nicotinic acetylcholine receptors α3 and α7 (**B**) increased significantly. Upper panels show representative Western blots for the markers and GAPDH. Lower panels show the densitometric values for these markers normalized to GAPDH. Values are means ± SE. n = 6 for each group. * $P < 0.05$, ** $P < 0.01$, versus control group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; α-SMA, α-smooth muscle actin.

the gonads to RGZ by itself did not result in any significant changes in global gonadal DNA methylation or in H3 and H4 acetylation (Figure 10).

Discussion

In the present series of experiments we have observed significant effects of nicotine treatment on lung function in the next two generations, affecting both the male and female offspring. In sharp contrast, nicotine treatment only affected the tracheal contractility of the male offspring. The functional effects of nicotine on the naive offspring were accompanied by increased expression of contractile proteins in the whole lung, as well as in the associated isolated lung fibroblasts, accompanied by decreased PPARγ expression. These nicotine-induced changes in lung function and mesenchymal protein expression, accompanied by decreased PPARγ expression, are consistent with the effect of nicotine on myofibroblast differentiation [32]. Even more importantly, along with the normalization of the asthma phenotype in the F1 and F2 offspring, most of the nicotine-induced lung and gonadal epigenetic changes were also normalized. For example, nicotine-induced increases in H3 acetylation in the lung, DNA methylation and H4 acetylation in the testis, and the decrease in DNA methylation and increase in H4 acetylation in the ovaries of F1 offspring were normalized by RGZ treatment, but it had no effect on lung H4 acetylation, providing further mechanistic specificity regarding the nature of the epigenetic mechanism. Given these insights, we will extend these studies to F3 and F4 generation offspring in future studies.

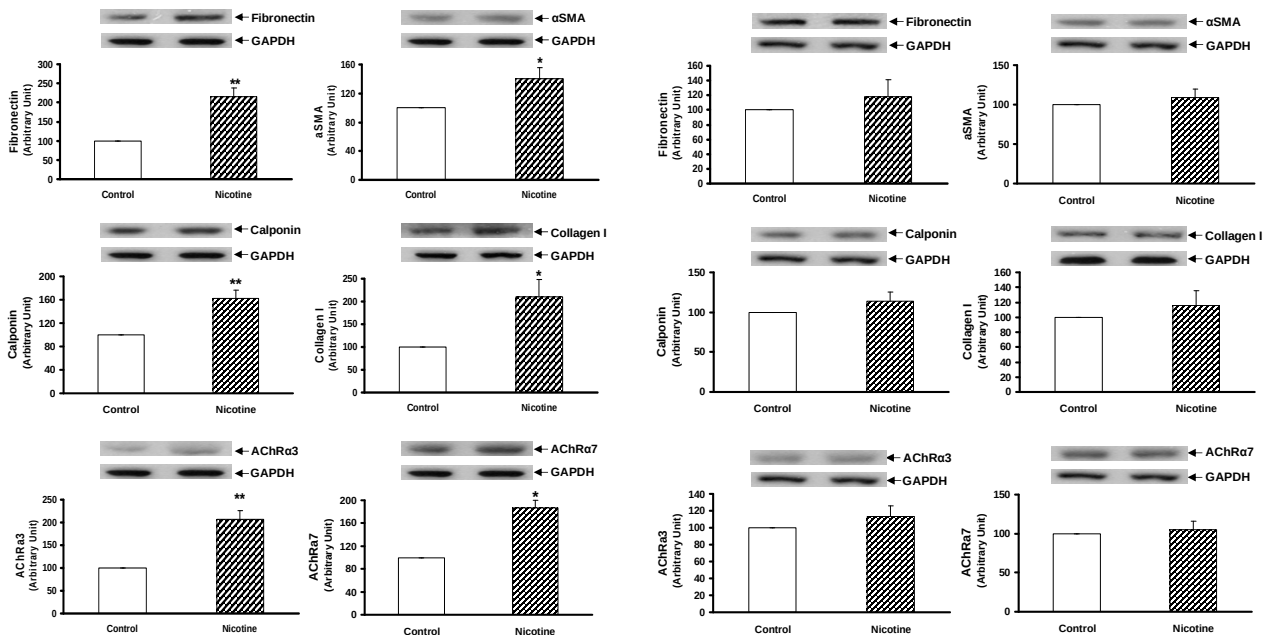

Figure 7 Effect of perinatal nicotine exposure on mesenchymal protein markers of airway reactivity and nicotinic acetylcholine receptors α3 and α7 in tracheas of male and female F2 rats. Compared to the control group, with nicotine administration the protein levels of fibronectin, α-SMA, calponin, and collagens I and III increased significantly in male (**A**) but not in female rats (**B**). Upper panels show representative Western blots for these markers and GAPDH. Lower panels show the densitometric values of these markers normalized to GAPDH. Values are means ± SE. n = 6 for each group. * $P < 0.05$, ** $P < 0.01$, versus control group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; α-SMA, α-smooth muscle actin.

Since ruling out genetic and environmental confounders is extremely difficult in humans, there is only scant evidence for MG epigenetic effects for any condition in humans [33]; and in fact, there is none for asthma. Recently emerging evidence suggests that the phenotype of an individual is the cumulative result of complex interactions between the genotype and its current, past and ancestral environments [27,34]. Therefore, it is logical to speculate a role for ancestral cigarette smoke exposure in the child's asthma predisposition. Yet, as pointed out above, there is no evidence for this, other than the data from the Children's Health Study from Southern California [17].

In contrast to other species, the evidence for fetal programming as a mode of MG transmission of traits in humans is very limited. For example, mothers from the Dutch Hunger Winter who were exposed to famine as fetuses delivered offspring of lower birth weights than those with no fetal exposure to famine, although this was not confirmed in a subsequent study [35]. There is also evidence of increased morbidity and mortality associated with parental and grandparental nutritional status, suggesting a role for fetal programming, possibly via epigenetic mechanisms to account for the MG effects [21,36,37]. In contrast to the very limited data in humans, in a variety of animal models gestational exposure to carcinogens, endocrine disruptors, and other toxins has been shown to have MG effects [38-40].

The present study is groundbreaking in our understanding of the mechanisms potentially involved in the transmission of epigenetic human diseases, which to date have only been speculated, albeit based on strong epidemiologic grounds [41]. The observation that nicotine exposure *in utero* affects the F1 offspring DNA methylation of the testes and ovaries, and that H3 acetylation was increased in the lungs and testes of F1 male offspring in association with male-specific increased tracheal constriction is the first demonstration of an epigenetic effect of nicotine on both gametocytes and somatocytes. Furthermore, the specific inhibition of this effect of nicotine on H3 acetylation by RGZ, which we have previously shown to block the nicotine-induced asthma phenotype in F1 offspring [20], provides a unique molecular genetic insight to the MG mechanism of nicotine action. It must be borne in mind, however, that these offspring's developing gonads had been exposed to nicotine *in utero*, leaving open the possibility that the effect was not a bona fide transgenerational effect, but was rather only MG. However, the transmission

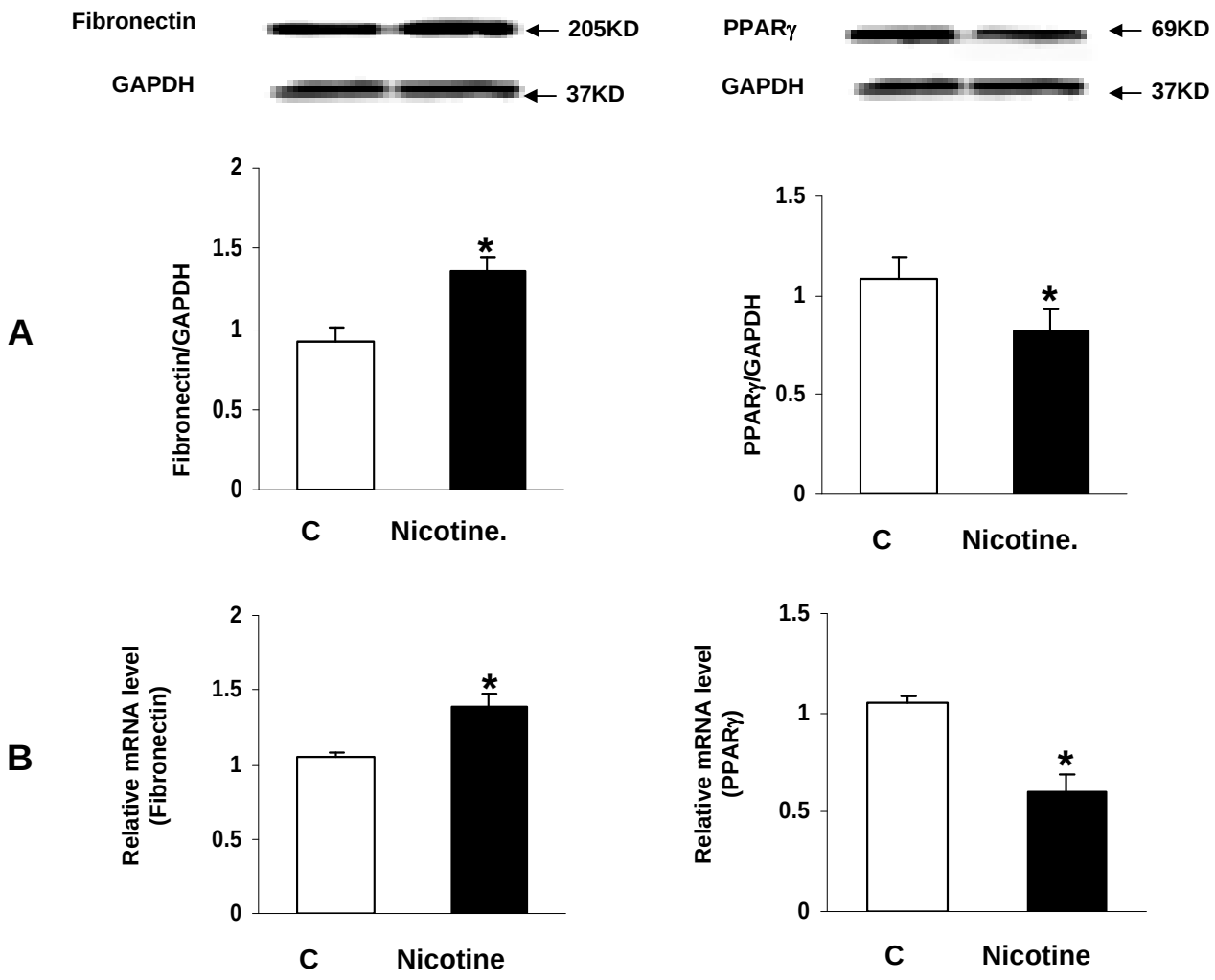

Figure 8 Effect of perinatal nicotine exposure on markers of differentiation in fibroblasts isolated from the lungs of F2 rats (mixed gender). Compared to the control group, with nicotine administration the expression of fibronectin increased and that of PPARγ decreased, at both the protein (**A**) and mRNA (**B**) levels. Values are means ± SE. n = 3 for each group. * $P < 0.05$, versus control group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PPARγ, peroxisome proliferator-activated receptor gamma.

of the asthma phenotype to the F2 generation, both structurally and functionally, and its prevention by a specifically-targeted molecular intervention is the first unequivocal demonstration of MG transmission of an epigenetically-mediated effect on the offspring phenotype. The fact that the phenotypic effect was on asthma, a well-recognized epidemiologic example of epigenetic transmission of the cause of a public health epidemic, makes this series of experiments all the more significant and noteworthy. This, and the recent finding that even 'thirdhand smoke' can induce the asthma phenotype [42], portends new and rational ways of thinking about effectively coping with the health hazards that abound all around us [43,44].

At first glance, it might seem surprising that the nicotine effect on the asthma phenotype is sex-specific. However, there is a documented association between gender and airway size, first referred to as dysanapsis by Mead [45], in which he showed that boys and women had self-similar airway structure that was distinctly different from that of men. Those data suggest that androgens may differentially affect airway development. We have previously shown that androgens affect the rate of lung development in rabbits and rats by inhibiting the glucocorticoid-induced differentiation of lung fibroblasts [46], consistent with their effect on lipofibroblast differentiation [47], which determines lung development [48]. The airway narrowing of dysanapsis has also been shown to be associated with asthma [49]. Therefore, androgens may precipitate asthma through a common genetic mechanism, since they, like nicotine, stimulate the Wnt pathway [50] and down-regulate PPARγ expression in lung fibroblasts [18-20,31,32].

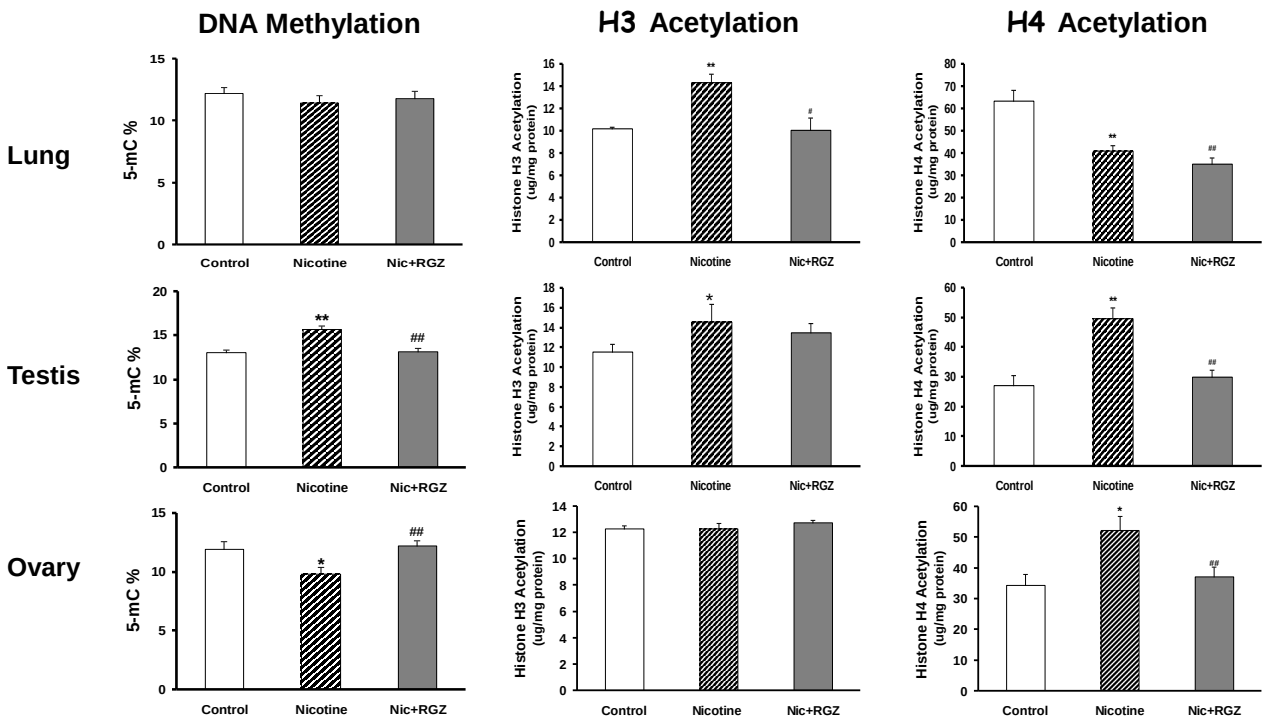

Figure 9 Effect of rosiglitazone (RGZ) in perinatal nicotine exposure-induced changes in global DNA methylation and histone (H) 3 and 4 acetylation in the lungs and gonads of F1 rats. Compared to the control group, with nicotine administration the level of global DNA methylation increased in the testes, decreased in the ovaries, but did not change in the lungs (**A**); H3 acetylation increased in the lungs and testes, but did not change in the ovaries (**B**); H4 acetylation decreased in the lungs, while it increased in the testes and ovaries (**C**). Most of these changes were blocked by concomitant RGZ administration. For example, in the lungs the nicotine-induced increase in lung H3 acetylation was blocked by RGZ, whereas there was no effect on global DNA methylation or on the nicotine-induced decrease in H4 acetylation; in the testes, the nicotine-induced increase in DNA methylation and H4 acetylation were blocked, without any effect on H3 acetylation; and in the ovaries, the nicotine-induced decrease in DNA methylation and increase in H4 acetylation were blocked without any effect on H3 acetylation. Values are means ± SE with variable numbers for each group. *$P < 0.05$, **$P < 0.01$, versus control; n = 4 to 6.

The compelling epigenetic data presented here potentially shift the current paradigm for our understanding of childhood asthma, and for the first time implicate epigenetics as the underlying cause for increased MG asthma following *in utero* exposure to maternal smoking. In fact, the data provided herein not only provide novel mechanistic information underlying the MG asthma risk, but also pave the way for studying the molecular mechanisms underlying MG effects for a host of other environmental toxins, agonists and antagonists as well.

It is important to point out that although there are many agents in cigarette smoke that may be detrimental to the developing lung, there is plenty of evidence to support nicotine as the main agent that alters fetal lung development: nicotine crosses the human placenta with minimal biotransformation [51]; it accumulates in fetal blood, maternal milk, amniotic fluid, and several fetal tissues, including the respiratory tract [52-54], and has been shown to have direct effects on pulmonary alveolar epithelial cells and interstitial fibroblasts isolated from the developing lung [32,43,55-60]. Therefore, it is not surprising that nicotine exposure during pregnancy is an extensively utilized and well-accepted model to study the effects of cigarette smoke on the developing lung in general, and on asthma in particular [18,20]. It is also important to emphasize that the main effects of *in utero* nicotine exposure on lung growth and development are due to specific alterations in signaling pathways involved in lung development [18,20,31,32,55-60], rather than being due to irreversible disruption by teratogenic or toxicological effects. This feature offers the opportunity for targeted interventions to modulate the effects of *in utero* nicotine exposure, in contrast to toxic or teratologic effects, which would be unlikely to be effectively blocked by any intervention.

It could be argued that the epigenetic marks in the gonads and lung are epiphenomena, and that the RGZ 'rescue' of the normal lung phenotype is an artifact. However, there is experimental evidence that methylation of H3/H4 results in down-regulation of PPARγ expression [61], mechanistically linking the epigenetic effect of nicotine with decreased lipofibroblast expression [18] and asthma [20].

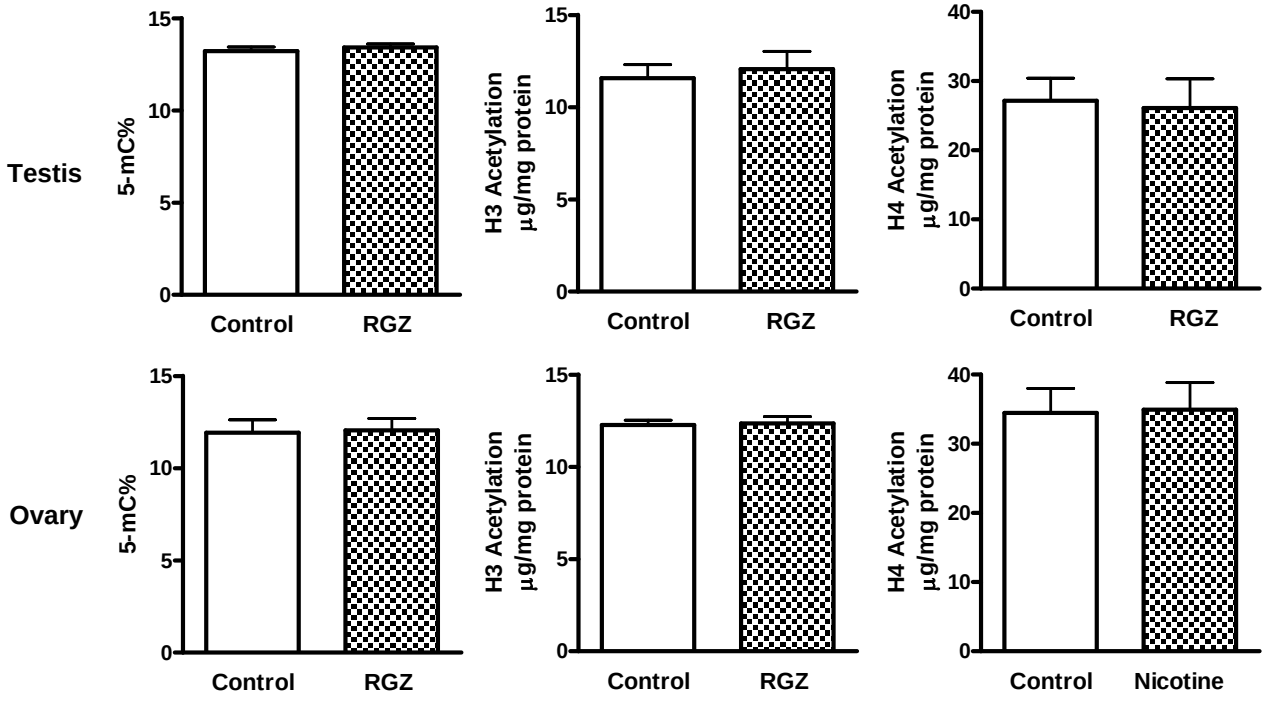

Figure 10 Effect of perinatal rosiglitazone exposure on global lung and gonadal DNA methylation and histone (H) 3 and 4 acetylation in F1 rats. With perinatal rosiglitazone exposure alone during gestation there was no effect on global DNA methylation and H3 and H4 acetylation in the lungs and gonads of F1 rat pups. Values are means ± SE; n = 4 to 6.

Conclusions

From the data included here we conclude that the pulmonary effects of nicotine exposure during pregnancy are not restricted only to the offspring of the exposed pregnancy, but are also transmitted to subsequent generation, possibly through germline epigenetic alterations, and, even more importantly, these effects can be blocked by targeted molecular interventions. Moreover, these data not only provide novel mechanistic information underlying the multigeneration transmission of asthma risk following exposure to maternal smoke during pregnancy, but also set precedence for studying other such environmental toxins that might have multigenerational or transgenerational effects.

Additional material

Additional File 1: Rat Model of Multigenerational Nicotine-induced Asthma. Rats were exposed to nicotine *in utero* (F0) to mimic maternal cigarette smoking. F0 offspring were then mated to generate F1 offspring, which in turn were mated to generate F2 offspring. Lungs, ovaries and testes are shown in black or white to symbolize direct or multigenerational nicotine exposure effects, respectively.

Abbreviations

BSA: bovine serum albumin; Cdyn: dynamic compliance; F0, F1, F2: parent, first, and second generations; FBS: fetal bovine serum; HAT: histone acetyl transferase; HBSS: Hanks' balanced salt solution; HDAC: histone deacetylase; H3, H4: histones 3 and 4; MEM: minimal essential medium; MG: multigenerational; PFT: pulmonary function test; PND: postnatal day; PPARγ: peroxisome proliferator activated receptor gamma; RGZ: rosiglitazone maleate; Rrs: lung resistance; RT-PCR: reverse transcriptase-polymerase chain reaction; α-SMA: α-smooth muscle actin.

Authors' contributions

VKR conceived the study, participated in study design, helped and coordinated the acquisition of data and its interpretation, and wrote the first draft the manuscript. JL carried out the animal work, molecular and pulmonary function studies, and made the data figures. EN and JT helped with DNA methylation and histone acetylation studies. RS performed fibroblast isolation and RT-PCR studies. KK helped with pulmonary function studies. OA supervised pulmonary function studies and the interpretation of pulmonary function data. JST helped in data interpretation, study design, and manuscript writing and revising. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

This study was supported in part by grants from the NIH (HL075405, HL55268, HD51857, HD058948, HL107118, and HD071731). However, this did not have any influence on study design, data collection, analysis and interpretation, and in writing and submission of the manuscript for publication.

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Received: 9 May 2012 Accepted: 30 October 2012
Published: 30 October 2012

References

1. Braman SS: **The global burden of asthma.** *Chest* 2006, **130**:4S-12S.
2. **Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee.** *Lancet* 1998, **351**:1225-1232.
3. Mannino DM, Homa DM, Akinbami LJ, Moorman JE, Gwynn C, Redd SC: **Surveillance for asthma–United States, 1980-1999.** *MMWR Surveill Summ* 2002, **51**:1-13.
4. Akinbami LJ, Moorman JE, Liu X: **Asthma prevalence, health care use, and mortality: United States, 2005-2009.** 2011, 1-12[http://www.cdc.gov/nchs/data/nhsr/nhsr032.pdf].
5. Smith DH, Malone DC, Lawson KA, Okamoto LJ, Battista C, Saunders WB: **A national estimate of the economic costs of asthma.** *Am J Respir Crit Care Med* 1997, **156**:787-793.
6. Adams PF, Hendershot GE, Marano MA: **Current estimates from the National Health Interview Survey, 1996.** *Vital Health Stat 10* 1999, 1-203.
7. Masoli M, Fabian D, Holt S, Beasley R: **The global burden of asthma: executive summary of the GINA Dissemination Committee report.** *Allergy* 2004, **59**:469-478.
8. Gilliland FD, Berhane K, McConnell R, Gauderman WJ, Vora H, Rappaport EB, *et al*: **Maternal smoking during pregnancy, environmental tobacco smoke exposure and childhood lung function.** *Thorax* 2000, **55**:271-276.
9. Haberg SE, Stigum H, Nystad W, Nafstad P: **Effects of pre- and postnatal exposure to parental smoking on early childhood respiratory health.** *Am J Epidemiol* 2007, **166**:679-686.
10. Hanrahan JP, Tager IB, Segal MR, Tosteson TD, Castile RG, Van VH, *et al*: **The effect of maternal smoking during pregnancy on early infant lung function.** *Am Rev Respir Dis* 1992, **145**:1129-1135.
11. Hofhuis W, de Jongste JC, Merkus PJ: **Adverse health effects of prenatal and postnatal tobacco smoke exposure on children.** *Arch Dis Child* 2003, **88**:1086-1090.
12. Moshammer H, Hoek G, Luttmann-Gibson H, Neuberger MA, Antova T, Gehring U, *et al*: **Parental smoking and lung function in children: an international study.** *Am J Respir Crit Care Med* 2006, **173**:1255-1263.
13. **March of Dimes. Alcohol and drugs.** 2010 [http://www.marchofdimes.com/pregnancy/alcohol_smoking.html].
14. **Federal Trade Commission. Federal Trade Commission Cigarette Report For 2002.** 2004 [http://ftc.gov/reports/cigarette/041022cigaretterpt.pdf].
15. **Centers For Disease Control and Prevention. Smoking & Tobacco Use.** [http://www.cdc.gov/tobacco/], 5-8-2012.
16. Martin JA, Hamilton BE, Sutton PD, Ventura SJ, Menacker F, Munson ML: **Births: final data for 2002.** *Natl Vital Stat Rep* 2003, **52**:1-113.
17. Li YF, Langholz B, Salam MT, Gilliland FD: **Maternal and grandmaternal smoking patterns are associated with early childhood asthma.** *Chest* 2005, **127**:1232-1241.
18. Rehan VK, Asotra K, Torday JS: **The effects of smoking on the developing lung: insights from a biologic model for lung development, homeostasis, and repair.** *Lung* 2009, **187**:281-289.
19. Krebs M, Sakurai R, Torday JS, Rehan VK: **Evidence for in vivo nicotine-induced alveolar interstitial fibroblast-to-myofibroblast transdifferentiation.** *Exp Lung Res* 2010, **36**:390-398.
20. Liu J, Sakurai R, O'Roark EM, Kenyon NJ, Torday JS, Rehan VK: **PPARgamma agonist rosiglitazone prevents perinatal nicotine exposure-induced asthma in rat offspring.** *Am J Physiol Lung Cell Mol Physiol* 2011, **300**:L710-L717.
21. Waterland RA, Jirtle RL: **Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases.** *Nutrition* 2004, **20**:63-8.
22. Jaenisch R, Bird A: **Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals.** *Nat Genet* 2003, **33(Suppl)**:245-54.
23. Waterland RA, Travisano M, Tahiliani KG: **Diet-induced hypermethylation at agouti viable yellow is not inherited transgenerationally through the female.** *FASEB J* 2007, **21**:3380-3385.
24. Cropley JE, Suter CM, Beckman KB, Martin DI: **Germ-line epigenetic modification of the murine A vy allele by nutritional supplementation.** *Proc Natl Acad Sci USA* 2006, **103**:17308-17312.
25. Pattenden S, Antova T, Neuberger M, Nikiforov B, De SM, Grize L, *et al*: **Parental smoking and children's respiratory health: independent effects of prenatal and postnatal exposure.** *Tob Control* 2006, **15**:294-301.
26. Alati R, Al MA, O'Callaghan M, Najman JM, Williams GM: **In utero and postnatal maternal smoking and asthma in adolescence.** *Epidemiology* 2006, **17**:138-144.
27. Gabory A, Attig L, Junien C: **Sexual dimorphism in environmental epigenetic programming.** *Mol Cell Endocrinol* 2009, **304**:8-18.
28. Zaren B, Lindmark G, Bakketeig L: **Maternal smoking affects fetal growth more in the male fetus.** *Paediatr Perinat Epidemiol* 2000, **14**:118-126.
29. Matta SG, Balfour DJ, Benowitz NL, Boyd RT, Buccafusco JJ, Caggiula AR, *et al*: **Guidelines on nicotine dose selection for in vivo research.** *Psychopharmacology (Berl)* 2007, **190**:269-319.
30. Rehan VK, Wang Y, Patel S, Santos J, Torday JS: **Rosiglitazone, a peroxisome proliferators-activated receptor γ agonist, prevents hyperoxia-induced neonatal rat lung injury in vivo.** *Pediatr Pulmonol* 2006, **41**:558-569.
31. Sakurai R, Cerny LM, Torday JS, Rehan VK: **Mechanism for nicotine-induced up-regulation of Wnt signaling in human alveolar interstitial fibroblasts.** *Exp Lung Res* 2011, **37**:144-154.
32. Rehan VK, Wang Y, Sugano S, Romero S, Chen X, Santos J, *et al*: **Mechanism of nicotine-induced pulmonary fibroblast transdifferentiation.** *Am J Physiol Lung Cell Mol Physiol* 2005, **289**:L667-L676.
33. Youngson NA, Whitelaw E: **Transgenerational epigenetic effects.** *Annu Rev Genomics Hum Genet* 2008, **9**:233-257.
34. Kabesch M: **Gene by environment interactions and the development of asthma and allergy.** *Toxicol Lett* 2006, **162**:43-48.
35. Lumey LH: **Decreased birthweights in infants after maternal in utero exposure to the Dutch famine of 1944-1945.** *Paediatr Perinat Epidemiol* 1992, **6**:240-253.
36. Ho DH, Burggren WW: **Epigenetics and transgenerational transfer: a physiological perspective.** *J Exp Biol* 2010, **213**:3-16.
37. Bygren LO, Kaati G, Edvinsson S: **Longevity determined by paternal ancestors' nutrition during their slow growth period.** *Acta Biotheor* 2001, **49**:53-59.
38. Kaati G, Bygren LO, Edvinsson S: **Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period.** *Eur J Hum Genet* 2002, **10**:682-688.
39. Pembrey ME, Bygren LO, Kaati G, Edvinsson S, Northstone K, Sjostrom M, *et al*: **Sex-specific, male-line transgenerational responses in humans.** *Eur J Hum Genet* 2006, **14**:159-166.
40. Skinner MK, Manikkam M, Guerrero-Bosagna C: **Epigenetic transgenerational actions of environmental factors in disease etiology.** *Trends Endocrinol Metab* 2010, **21**:214-222.
41. Christensen BC, Marsit CJ: **Epigenomics in environmental health.** *Front Genet* 2011, **2**:84.
42. Rehan VK, Sakurai R, Torday JS: **Thirdhand smoke: a new dimension to the effects of cigarette smoke on the developing lung.** *Am J Physiol Lung Cell Mol Physiol* 2011, **301**:L1-L8.
43. Wigle DT, Arbuckle TE, Turner MC, Berube A, Yang Q, Liu S, *et al*: **Epidemiologic evidence of relationships between reproductive and child health outcomes and environmental chemical contaminants.** *J Toxicol Environ Health B Crit Rev* 2008, **11**:373-517.
44. Skinner MK: **Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability.** *Epigenetics* 2011, **6**:838-842.
45. Mead J: **Dysanapsis in normal lungs assessed by the relationship between maximal flow, static recoil, and vital capacity.** *Am Rev Respir Dis* 1980, **121**:339-342.
46. Torday J: **Cellular timing of fetal lung development.** *Semin Perinatol* 1992, **16**:130-139.
47. Singh R, Artaza JN, Taylor WE, Gonzalez-Cadavid NF, Bhasin S: **Androgens stimulate myogenic differentiation and inhibit adipogenesis in C3H 10T1/2 pluripotent cells through an androgen receptor-mediated pathway.** *Endocrinology* 2003, **144**:5081-5088.
48. Torday JS, Torres E, Rehan VK: **The role of fibroblast transdifferentiation in lung epithelial cell proliferation, differentiation, and repair in vitro.** *Pediatr Pathol Mol Med* 2003, **22**:189-207.
49. Simon DM, Tsai LW, Ingenito EP, Starcher BC, Mariani TJ: **PPARgamma deficiency results in reduced lung elastic recoil and abnormalities in airspace distribution.** *Respir Res* 2010, **11**:69.
50. Liu XH, Kirschenbaum A, Yao S, Liu G, Aaronson SA, Levine AC: **Androgen-induced Wnt signaling in preosteoblasts promotes the growth of MDA-PCa-2b human prostate cancer cells.** *Cancer Res* 2007, **67**:5747-5753.

51. Luck W, Nau H: **Nicotine and cotinine concentrations in serum and milk of nursing smokers.** *Br J Clin Pharmacol* 1984, **18**:9-15.
52. Dempsey D, Jacob P III, Benowitz NL: **Accelerated metabolism of nicotine and cotinine in pregnant smokers.** *J Pharmacol Exp Ther* 2002, **301**:594-598.
53. Luck W, Nau H, Hansen R, Steldinger R: **Extent of nicotine and cotinine transfer to the human fetus, placenta and amniotic fluid of smoking mothers.** *Dev Pharmacol Ther* 1985, **8**:384-395.
54. Szuts T, Olsson S, Lindquist NG, Ullberg S, Pilotti A, Enzell C: **Long-term fate of [14C]nicotine in the mouse: retention in the bronchi, melanin-containing tissues and urinary bladder wall.** *Toxicology* 1978, **10**:207-220.
55. Maritz GS, Thomas RA: **Maternal nicotine exposure: response of type II pneumocytes of neonatal rat pups.** *Cell Biol Int* 1995, **19**:323-331.
56. Roman J, Ritzenthaler JD, Gil-Acosta A, Rivera HN, Roser-Page S: **Nicotine and fibronectin expression in lung fibroblasts: implications for tobacco-related lung tissue remodeling.** *FASEB J* 2004, **18**:1436-1438.
57. Rehan VK, Wang Y, Sugano S, Santos J, Patel S, Sakurai R, *et al*: **In utero nicotine exposure alters fetal rat lung alveolar type II cell proliferation, differentiation, and metabolism.** *Am J Physiol Lung Cell Mol Physiol* 2007, **292**:L323-L333.
58. Sekhon HS, Jia Y, Raab R, Kuryatov A, Pankow JF, Whitsett JA, *et al*: **Prenatal nicotine increases pulmonary alpha7 nicotinic receptor expression and alters fetal lung development in monkeys.** *J Clin Invest* 1999, **103**:637-647.
59. Sekhon HS, Keller JA, Proskocil BJ, Martin EL, Spindel ER: **Maternal nicotine exposure upregulates collagen gene expression in fetal monkey lung. Association with alpha7 nicotinic acetylcholine receptors.** *Am J Respir Cell Mol Biol* 2002, **26**:31-41.
60. Joad JP, Ji C, Kott KS, Bric JM, Pinkerton KE: **In utero and postnatal effects of sidestream cigarette smoke exposure on lung function, hyperresponsiveness, and neuroendocrine cells in rats.** *Toxicol Appl Pharmacol* 1995, **132**:63-71.
61. Tsukamoto H, Zhu NL, Asahina K, Mann DA, Mann J: **Epigenetic cell fate regulation of hepatic stellate cells.** *Hepatol Res* 2011, **41**:675-682.

Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1741-7015/10/129/prepub

doi:10.1186/1741-7015-10-129
Cite this article as: Rehan *et al.*: Perinatal nicotine exposure induces asthma in second generation offspring. *BMC Medicine* 2012 **10**:129.

Am J Physiol Lung Cell Mol Physiol 305: L501–L507, 2013.
First published August 2, 2013; doi:10.1152/ajplung.00078.2013.

Perinatal nicotine-induced transgenerational asthma

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Submitted 22 March 2013; accepted in final form 29 July 2013

Rehan VK, Liu J, Sakurai R, Torday JS. Perinatal nicotine-induced transgenerational asthma. *Am J Physiol Lung Cell Mol Physiol* 305: L501–L507, 2013. First published August 2, 2013; doi:10.1152/ajplung.00078.2013.—Asthma is a major public health hazard worldwide. Its transgenerational inheritance has been inferred from epidemiological studies. More recently, using nicotine as a proxy for maternal smoking, we have demonstrated that an asthma-like phenotype can be inherited by rat offspring for up to two generations, i.e., multigenerationally, after the initial intrauterine exposure. We hypothesized that asthma transmission to offspring following perinatal nicotine exposure is not restricted up to F_2 generation, but it also extends to subsequent generations. To test this hypothesis, using a well-established rat model of nicotine exposure-induced childhood asthma, we determined if perinatal nicotine exposure of F_0 gestating dams would transmit asthma transgenerationally to F_3 offspring. We now extend our findings to third-generation offspring, including abnormal pulmonary function, particularly as it relates to the occurrence in the upper airway exclusively in males, and to its effects on molecular functional markers (fibronectin and peroxisome proliferator-activated receptor γ), previously shown to be consistent with the asthma phenotype, herein expressed in fibroblasts isolated from the lung. These data, for the first time, demonstrate the transgenerational transmission of the asthma phenotype to F_3 offspring following perinatal nicotine exposure of F_0 dams.

peroxisome proliferator-activated receptor γ; maternal smoking; pulmonary function

ASTHMA IS A MAJOR PUBLIC HEALTH problem, with an estimated 300 million people affected worldwide, and this number is projected to increase to 400 million by 2025 (1, 25). Although a multitude of causes contribute to childhood asthma, maternal smoking during pregnancy is a well-established contributor (6, 7, 9, 10, 23, 30), being a major modifiable risk factor, the elimination of which could significantly reduce the prevalence of childhood asthma. The recognition that childhood asthma is induced by smoke exposure during pregnancy is particularly important since there is emerging evidence that, following in utero exposure to maternal smoke, asthma can be transmitted multigenerationally (18). Yet, up until our recent demonstration of the multigenerational transmission of asthma in an animal model (35), there was neither experimental evidence nor any mechanistic explanation for this phenomenon. Using a well-established rat model of in utero nicotine exposure for childhood asthma (12, 13, 19, 20, 24, 34), we recently demonstrated that, following perinatal exposure to nicotine during F_0 pregnancy (17, 35), asthma can be inherited by rat offspring up to the F_2 generation, i.e., multigenerationally. Now, we hypothesize that asthma transmission to offspring following perinatal nicotine exposure is not restricted up to F_2 generation, but it also extends to subsequent generations. Using our rat model of perinatal nicotine exposure-induced childhood asthma (16, 19, 20, 34, 35), in this study we aimed to determine if perinatal nicotine exposure of F_0 gestating dams would transmit asthma transgenerationally to F_3 offspring.

For clarification, it is important to point out the difference between multigenerational vs. transgenerational (TG) transmission of an acquired trait. Transmission of a trait from the gestating F_0 dam up to F_2 generation offspring, in response to an environmental exposure, for example, asthma in the case of in utero nicotine exposure, constitutes multigenerational inheritance; however, such inheritance is not "transgenerational" since the transmission of the acquired trait is likely to be due to the direct exposure of the F_2 generation germline to the environmental challenge during the F_0 gestation. In contrast, transmission of an induced trait following exposure to an environmental challenge during the F_0 generation out to the F_3 generation, the generation that's not directly exposed to the environmental challenge, constitutes TG inheritance such as that reported in this manuscript.

METHODS

Materials. Nicotine bitartrate was acquired from Sigma-Aldrich (St. Louis, MO). All plasticware and culture media were purchased from Corning (Corning, NY) and Invitrogen (San Diego, CA).

The animal model. Time-mated, first-time pregnant, pair-fed Sprague Dawley rat dams (F_0) received either placebo (diluent) or nicotine (1 mg/kg sc) in 100-μl volumes daily from *embryonic day 6* of gestation to *postnatal day* (PND) *21*. Following delivery at term, the F_1 pups were allowed to breast feed ad libitum. At PND21, the pups were subjected to pulmonary function tests, tracheal tension and airway contractility studies, and mesenchymal differentiation mRNA and protein level [fibronectin and peroxisome proliferator-activated receptor γ (PPARγ)] determinations, as described below. Some F_1 male and female littermates were weaned at PND21 and maintained in separate cages as breeders to generate F_2 rats, but without any subsequent exposure to nicotine in the F_1 or F_2 pregnancies. F_3 pups were similarly obtained, using F_2 offspring as breeders. At PND21, F_3 pups underwent studies similar to those performed on F_1 rats.

Pulmonary function testing. Measurement of respiratory function was performed using plethysmography for restrained animals (Buxco, Troy, NY) as described by us previously (19, 35). Briefly, the pups were deeply anesthetized and sedated with ketamine (70 mg/kg; Bioniche Teoranta Inverin, Galway, Ireland) and xylazine (7 mg/kg; Akorn, Decatur, IL), tracheostomized, and ventilated. Next, the pups were exposed to increasing concentrations of aerosolized methacholine (0, 1.25, 2.5, 5, 10, and 20 mg/ml) over a period of 3 min each, and lung resistance (Rrs) and dynamic compliance (Cdyn) were subsequently measured and plotted as a function of the methacholine concentration administered.

Tracheal tension studies. The whole trachea was excised immediately after death and dissected free of connective tissue in ice-cold modified Krebs-Ringer bicarbonate buffer (expressed as mM concentrations: 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4,

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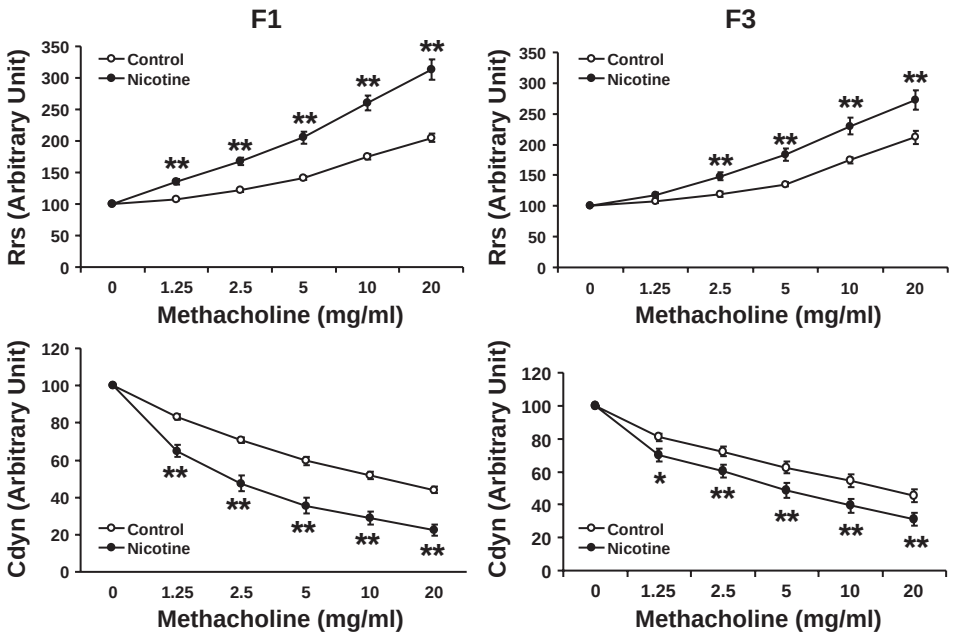


Fig. 1. Effect of perinatal nicotine exposure on total respiratory system resistance and compliance in F_1 and F_3 rats. Compared with the control group, with nicotine administration to rat dams there was a significant increase in total airway resistance (Rrs) and a decrease in total compliance (Cdyn) following methacholine (Mch) challenge in both F_1 (*left*) and F_3 (*right*) rat offspring of mixed gender, even though the F_3 rats were not exposed to any nicotine during the F_1 and F_2 gestations. Values are means ± SE; $n = 10–12$ rats for each group. $*P < 0.05$ and $**P < 0.01$ vs. control.

25.0 $NaHCO_3$, and 11.1 glucose). Approximately a 6-mm tracheal ring was resected from the midsection of each trachea and suspended in an organ chamber containing 10 ml of modified Krebs-Ringer bicarbonate buffer solution maintained at 37 ± 0.5°C and aerated with 95% O_2-5% CO_2 (pH 7.4). Each ring was suspended via two stirrups, each passed through the lumen: one stirrup was anchored to the bottom of the organ chamber, and the other stirrup was connected to a strain gauge (model FT03C; Grass Instrument, Quincy, MA) for the measurement of isometric force, as described previously (5).

For tracheal tension measurements, each tracheal ring was initially stretched to its optimal resting tension, which was achieved by stepwise stretching in 0.1-g increments, until the contractile response to 100 mM KCl reached a plateau. Each tracheal ring was allowed to equilibrate for 1 h, after which the effect of acetylcholine was determined at least 30 min after the administration of nitro-L-arginine (1×10^{-4} M, an inhibitor of nitric oxide synthase). In all experiments, indomethacin (1×10^{-5} M) was added to the bath to prevent possible interference by prostanoids.

Lung fibroblast isolation. PND21 rat lung fibroblasts from both F_1 and F_3 generation pups were cultured using slight modifications of our previously described methods (40, 41). Briefly, the lungs were trimmed to remove major airways and rinsed with calcium- and magnesium-free Hanks' balanced salt solution. Lung tissue was minced into 1- to 2-mm^3 pieces and was suspended in prewarmed (37°C) digestion buffer containing 2.5 ml of heat-inactivated chicken serum (2.5 ml), HEPES (1.25 ml at 500 mM, pH 7.4), collagenase I (12.5 mg; Sigma Chemical), and collagenase 1A (12.5 mg; Sigma Chemical) in Waymouth's medium (in a final volume of 25 ml). The tissue was triturated 100 times with a 10-ml pipette, 100 times with a 5-ml pipette, and 100 times with a 9-in. Pasteur pipette. The tissue was

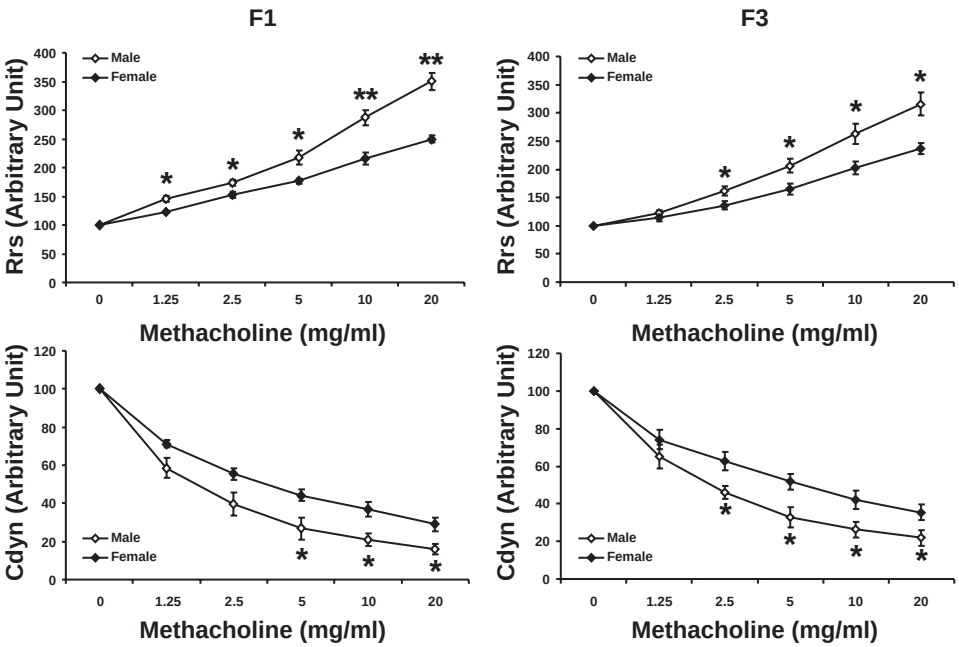


Fig. 2. Effect of perinatal nicotine exposure on total respiratory system resistance and compliance in F_1 and F_3 male and female rats. Compared with the control group, nicotine treatment significantly increased Rrs and decreased Cdyn of the lung following methacholine (Mch) challenge in F_1 (*left*) and F_3 (*right*) male (open diamonds) and female (closed diamonds) rats even though the F_1 and F_2 pups were not exposed to nicotine during gestation. Values are means ± SE; $n = 5–6$ for each group. $*P < 0.05$ and $**P < 0.01$ vs. control.

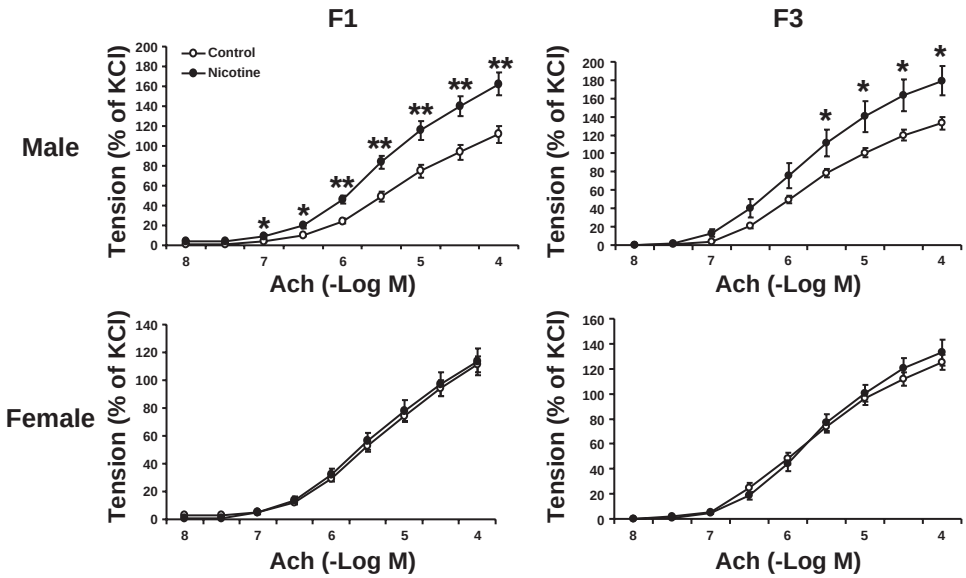


Fig. 3. Effect of perinatal nicotine exposure on tracheal constriction response to acetylcholine in F_1 and F_3 male and female rats. Compared with the control group, with perinatal nicotine administration there was a significant increase in tracheal constriction in response to acetylcholine only in males (*top*) and not in females (*bottom*) in both F_1 (*left*) and F_3 (*right*) rats. Values are means ± SE; $*P < 0.05$ and $**P < 0.01$ vs. the control group; $n = 4$.

further dissociated in a 37°C water bath using a Teflon stirring bar to disrupt the tissue mechanically. Once the tissue was dispersed in a unicellular suspension, the cells were pelleted at 500 *g* for 10 min at room temperature in a 50-ml polystyrene centrifuge tube. The supernatant was decanted, and the pellet was resuspended in Minimal Essential Medium containing 20% fetal bovine serum to yield a mixed cell suspension of ca. 3×10^8 cells, as determined with a Coulter particle counter (Beckman-Coulter, Hialeah, FL). The cell suspension was then added to tissue culture flasks (75 cm^2) for 30–60 min to allow for differential adherence of the lung fibroblasts. These cells are greater than 95% pure fibroblasts based upon their morphological appearance when viewed at the light microscopic level and by immunohistochemical staining for vimentin.

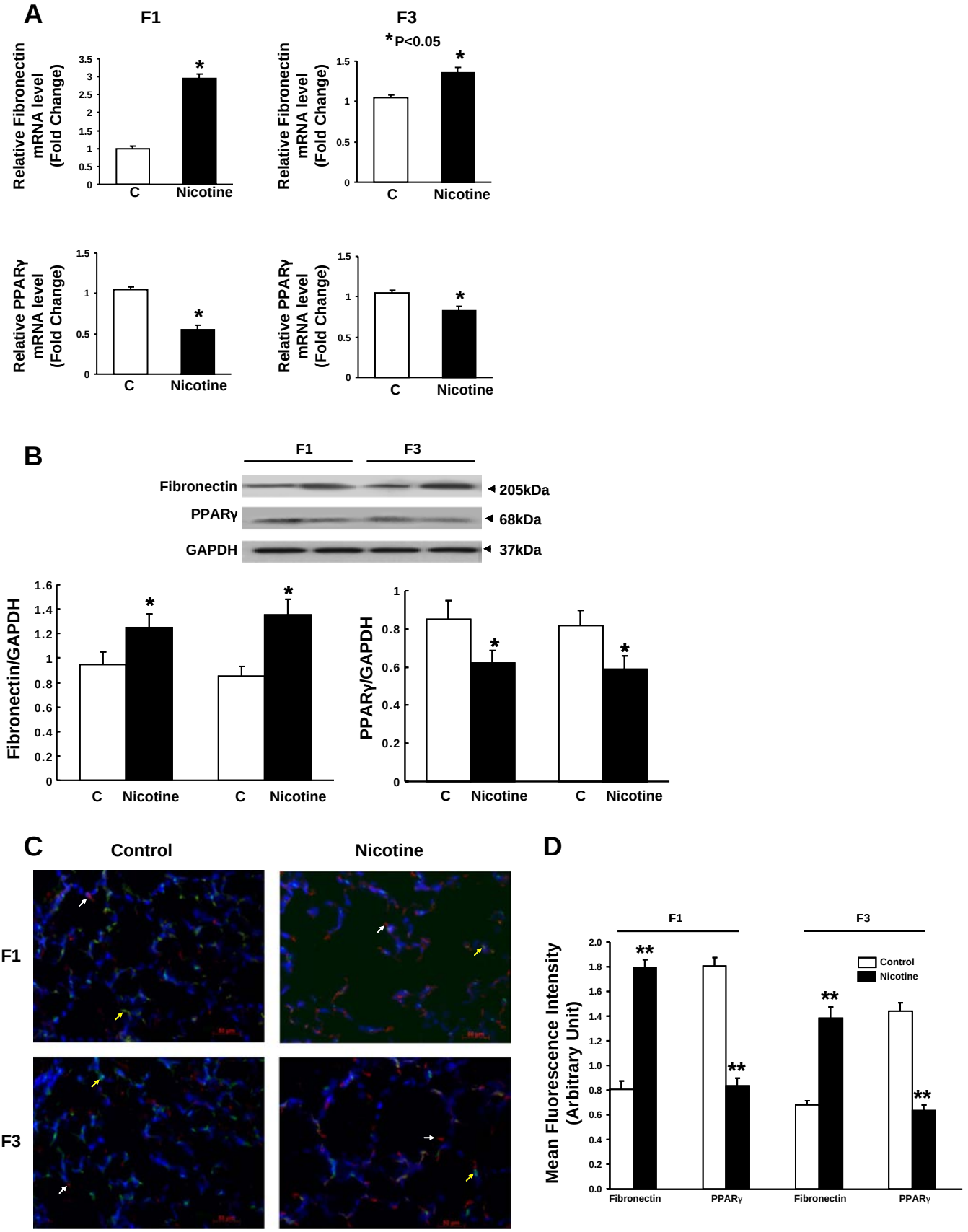
Western analysis. Western analysis on protein lysates from cultured lung fibroblasts from F_1 and F_3 generation pups was performed according to previously described methods (37). The protein concentration of the supernatant was measured by the Bradford method, using bovine serum albumin as the standard. Aliquots of the supernatant, each containing 30 μg of protein, were separated by SDS-PAGE gel and electrically transferred to nitrocellulose membranes. Nonspecific binding sites were blocked by incubation with Tris-buffered saline (TBS) containing 5% nonfat dry powdered milk (wt/vol) for 1 h at room temperature. After a brief rinse with TBS containing 0.1% Tween 20 (TBST), the protein blots were incubated in primary antibody (PPARγ, 1:500, Santa Cruz, catalog no. sc-7196; fibronectin, 1:1,000, BD Biosciences, catalog no. 610078; or GAPDH, 1:4,000, Millipore, catalog no. MAB374) overnight at 4°C followed by incubation with an appropriate secondary antibody for 1 h at room temperature. After three more washes in TBST, the blots were exposed to X-ray film using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) and developed. The relative densities of the protein bands were determined with UN-SCAN-IT software (Silk Scientific, Orem, UT) and normalized to that of GAPDH.

Real-time RT-PCR. In brief, total RNA was isolated using an RNAqueous-4PCR kit (Ambion) and was DNase-treated and quantitated by light absorbance using a Nanodrop spectrophotometer (Nanodrop Instruments, Wilmington, DE). The structural integrity of the RNA was assessed based on the visual appearance of the ethidium bromide-stained ribosomal bands following fractionation on a 1.2% (wt/vol) agarose-formaldehyde gel and quantitated by light absorbance at 260 nm. Total RNA (1 μg) was reverse-transcribed into single-stranded cDNA using a TaqMan Gold RT-PCR Kit at 50°C for 30 min in a total volume of 20 μl. The PCR reaction mix consisted of 1 μl of 10-fold diluted cDNA and PCR Gold DNA polymerase reagent mix, and optimized for forward and reverse gene-specific primers (900 nM each) with a gene-specific probe (250 nM, FAM dye label). Primer sets were purchased predesigned (TaqMan Gene Expression Assays; Applied Biosystems). Real-Time PCR reactions were run in triplicate on 96-well plates using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Reactions proceeded by activation of DNA polymerase at 95°C for 10 min, followed by 38 PCR denaturing cycles at 95°C for 15 s and annealing/extension at 60°C for 1 min. Data were normalized to 18S ribosomal RNA using an RNA TaqMan Gene Expression Assay and were analyzed to select a threshold level of fluorescence that was in the linear phase for PCR product accumulation [the threshold cycle (C_T) for that reaction]. The C_T value for 18S ribosomal RNA was subtracted from the C_T value for the gene of interest to obtain a delta C_T (ΔC_T) value. The relative fold-change for each gene was calculated using the $\Delta\Delta C_T$ method. Results are expressed as means ± SE and considered significant at $P < 0.05$. RT-PCR probes used included rat PPARγ: forward-5′-CCAAGTGACTCTGCTCAAGTATGG-3′ and reverse-5′-CATGAATCCTTGTCCCTCTGATATG-3′ (106 bp); rat fibronectin: forward-5′-AGCACACCCGTTTTCATCCA-3′ and reverse-5′-TTTCACGTCGGTCACTTCCA; and rat 18s: 5′-TTAAGCCATGCATGTCTAAGTAC and 3′-TGTTATTTTTCGTCACTACCTCC.

Immunofluorescence staining. Rat lungs were inflation fixed in situ with 4% paraformaldehyde in phosphate buffer at a standard inflation pressure of 5 cm of H_2O. Fibronectin and PPARγ protein expression were assessed by double-immunofluorescence staining. Briefly, 5-μm sections were incubated with a mouse monoclonal antibody against fibronectin (1:500 dilution, catalog no. 610078; BD Biosciences, San Jose, CA) and a rabbit polyclonal antibody against PPARγ (1:50 dilution, catalog no. SC-7196; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight and then incubated with Alexa Fluor 594 goat

Table 1. *Gender composition in Figure 1*

Generation	Group	Male	Female	Total No.
F_1	Control	6	6	12
	Nicotine	6	6	12
F_3	Control	7	6	13
	Nicotine	7	6	13



anti-mouse IgG (1:500 dilution, catalog no. A31624 for fibronectin; Invitrogen, Carlsbad, CA), and Alexa Fluor 488 goat anti-rabbit IgG (1:50 dilution, catalog no. A31628 for PPARγ; Invitrogen) was applied to the sections for 1 h at room temperature. The sections were washed with phosphate-buffered saline and then mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) for visualization under a fluorescence microscope.

Statistics. The data for analysis were obtained from at least three independent sets of experiments. Analysis of variance for multiple comparisons with Bonferroni post hoc analysis and Student's *t*-test were used as indicated, and $P < 0.05$ was considered to indicate statistically significant differences among the experimental groups.

RESULTS

We initially determined the effects of perinatal nicotine exposure on total Rrs and Cdyn in F_3 generation rats at PND21 following a methacholine challenge and compared these data with our previously published data in F_1 generation rats [please note that F_1 generation data included here for comparison with F_3 generation data have been published previously (35)]. Similar to the F_1 generation data, compared with the control group, with perinatal nicotine exposure only to F_0 dams, there was a significant increase in Rrs and a decrease in Cdyn of the total respiratory system following the methacholine challenge in F_3 rats, even though the F_3 rats were not exposed to any nicotine either during gestation or postnatally (Fig. 1). After establishing this experimental evidence for the TG transmission of perinatal nicotine exposure-induced asthma, we then examined whether this TG transmission of asthma was gender specific. We found that, compared with controls, with perinatal nicotine exposure only to F_0 dams, the total airway Rrs increased, and the total airway Cdyn decreased in both the males and females in both the F_1 and F_3 generations, but these changes were significantly greater in the males than the females in both generations (Fig. 2). Because we previously found that in the F_1 generation the effect of perinatal nicotine exposure on the tracheal constriction response was gender specific, i.e., seen exclusively in males (19), we next determined if the same was true for the F_3 generation and found a similar differential gender effect in the F_3 generation as well (Fig. 3). It is important to point out that the gender composition and male-to-female ratios of the F_1 and F_3 animals studied were similar (Table 1). To determine the potential mechanism of airway hyperresponsiveness to the methacholine challenge, we next determined the expression of airway contractility and differentiation markers by the lung fibroblasts isolated from the F_3 generation rats. Similar to our findings for the changes in the levels of these markers in F_1 rat lungs, as determined by Real-Time RT-PCR and Western analysis (19, 20), both fibronectin mRNA and protein levels were increased, and PPARγ mRNA and protein levels were decreased in cultured fibroblasts from F_3 rats (Fig. 4, *A* and *B*), providing further evidence for the nicotine-induced TG transmission of the lung cellular/molecular phenotype from the F_1 through the F_3 generation. The upregulation of fibronectin and downregulation of PPARγ protein levels in both F_1 and F_3 generation rat lungs was also corroborated by immunofluorescence staining of lung sections for these same proteins (Fig. 4, *C* and *D*).

DISCUSSION

We have previously observed significant effects of nicotine treatment on lung function in generations F_1 and F_2, constituting "multigenerational" inheritance (17, 35). In the present study, using the same experimental approach, we now document a TG effect of perinatal nicotine exposure on lung function for the first time, i.e., F_1–F_3. As in the previous study, we have observed significant effects of nicotine treatment on lung function in the F_3 generation, affecting both the male and female offspring and nicotine treatment only affecting the tracheal contractility of the male offspring. These functional effects of nicotine were again accompanied by increased expression of the myogenic protein fibronectin but decreased expression of PPARγ in the isolated lung fibroblasts, consistent with the effect of nicotine on myofibroblast differentiation (42, 43).

Our previously reported study of the multigenerational effect of maternal nicotine treatment on the asthma phenotype was groundbreaking in our understanding of the putative mechanisms involved in the transmission of epigenetic human disease, which to date has only been speculated, albeit based on strong epidemiological grounds (3). The fact that in this instance the phenotypic effect was on an asthma-like phenotype, a well-recognized epidemiological example for the epigenetic transmission of the cause of a public health epidemic, makes this series of experiments all the more significant and compelling, heralding a new and rational way of more effectively coping with the rising asthma epidemic (1, 25, 28, 39, 45). Moreover, since we had found that in our previous study of F_1 and F_2 generation pups most of the nicotine-induced lung and gonadal epigenetic changes were normalized by treatment with the PPARγ agonist rosiglitazone, and since we have observed decreased PPARγ expression in the F_3 generation, we predict that its upregulation will normalize the asthma-like phenotype here too. Furthermore, in line with our previous data in F_1 offspring, there was a gender-specific tracheal contractility response in F_3 offspring as well, with increased nicotine exposure-induced contractility seen only in the males. Although the mechanism underlying this phenomenon remains to be determined, we speculate that it might reflect differential upregulation of Wnt signaling in the male upper airway, as has been previously observed in F_1 generation offspring (19).

The evidence for fetal programming as a mode of TG transmission of phenotypic traits in humans is limited, for example, mothers from the Dutch Hunger Winter who were exposed to famine as fetuses delivered offspring of lower birth

Fig. 4. Effect of perinatal nicotine exposure in F_0 generation on the levels of mesenchymal markers of airway differentiation in lung fibroblasts derived from F_1 and F_3 rats. Compared with controls, with nicotine exposure to F_0 dams, mRNA (*A*) and protein (*B*) levels of fibronectin increased, whereas that of peroxisome proliferator-activated receptor γ (PPARγ) decreased in fibroblasts isolated from F_1 and F_3 rat lungs. Values are means ± SE; $n = 3$. *$P < 0.05$ vs. control. The upregulation of fibronectin (red staining, white arrows) and downregulation of PPARγ (green staining, yellow arrows) protein levels in both F_1 and F_3 generation rat lungs were corroborated by immunofluorescence staining of lung sections for these proteins (*C*). The mean fluorescence intensity for fibronectin and PPARγ staining of 6 comparable lung fields from each group, quantified using ImageJ software, is shown (*D*). Values are means ± SE. **$P < 0.01$ vs. the control group; $n = 3$.

weight than those with no fetal exposure to famine (21). There is also evidence for increased morbidity and mortality associated with parental and grandparental nutritional status, suggesting a role for fetal programming, possibly via epigenetic mechanisms, accounting for the TG effects (2, 14, 32).

In contrast to such limited human data, in a variety of animal models gestational exposure to carcinogens, endocrine disruptors, or other toxins has been claimed to have TG effects; however, most of these studies have not determined such effects beyond the second generation (22, 27, 36, 46). Some examples are the multigenerational effect of overfeeding causing the diabetic phenotype (33), prenatal exposure to allergens (31), and hepatotoxicity of carbon tetrachloride (47). It is noteworthy that the latter model strongly parallels our findings for the generational effects of nicotine, since hyperactive myofibroblasts are implicated in the pathobiology of both asthma and liver fibrosis. However, the only valid examples of TG inheritance in the literature are those of an altered stress response (4), and multiple structural and functional alterations (22) in F_3 rat offspring exposed to either vinclozolin (4), or plastic-derived endocrine disruptors such as bisphenol-A, bis(2-ethylhexyl)phthalate, or dibutyl phthalate to gestating F_0 females (22), respectively.

The compelling data presented herein provide the first experimental evidence for TG transmission of an asthma-like phenotype following in utero exposure to maternal smoking, potentially shifting the current paradigm for our understanding of childhood asthma. These data pave the way for determining the epigenetic mechanisms, such as DNA methylation, histone modifications, and noncoding RNA production, likely underlying in utero smoke exposure-induced TG transmission of asthma (8, 11, 26, 29, 44).

GRANTS

This study was supported in part by National Institutes of Health Grants HL-075405, HL-55268, HD-51857, HD-058948, HL-107118, and HD-071731.

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

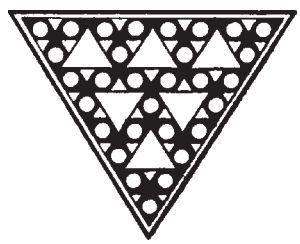
AUTHOR CONTRIBUTIONS

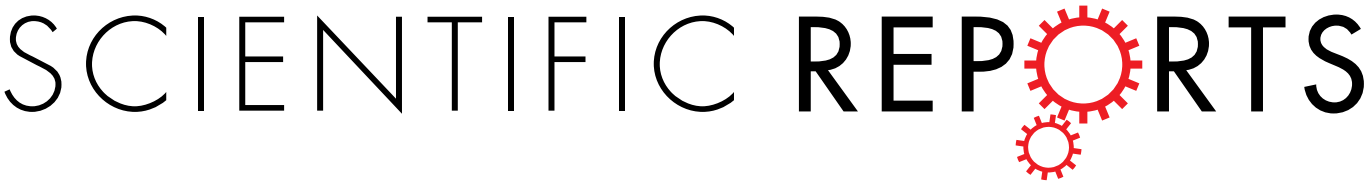
Author contributions: V.K.R. conception and design of research; V.K.R. and J.L. analyzed data; V.K.R. and J.S.T. interpreted results of experiments; V.K.R. drafted manuscript; V.K.R. and J.S.T. edited and revised manuscript; V.K.R., J.L., R.S., and J.S.T. approved final version of manuscript; J.L. and R.S. performed experiments; J.L. and R.S. prepared figures.

REFERENCES

1. **Braman SS.** The global burden of asthma. *Chest* 130, *Suppl* 1: 4S–12S, 2006.
2. **Bygren LO, Kaati G, Edvinsson S.** Longevity determined by paternal ancestors' nutrition during their slow growth period. *Acta Biotheor* 49: 53–59, 2001.
3. **Christensen BC, Marsit CJ.** Epigenomics in environmental health (Abstract). *Front Genet* 2: 84, 2011.
4. **Crews D, Gillette R, Scarpino SV, Manikkam M, Savenkova MI, Skinner MK.** Epigenetic transgenerational inheritance of altered stress responses. *Proc Natl Acad Sci USA* 109: 9143–9148, 2012.
5. **Gao Y, Dhanakoti S, Trevino EM, Sander FC, Portugal AM, Raj JU.** Effect of oxygen on cyclic GMP-dependent protein kinase-mediated relaxation in ovine fetal pulmonary arteries and veins. *Am J Physiol Lung Cell Mol Physiol* 285: L611–L618, 2003.
6. **Gilliland FD, Berhane K, McConnell R, Gauderman WJ, Vora H, Rappaport EB, Avol E, Peters JM.** Maternal smoking during pregnancy, environmental tobacco smoke exposure and childhood lung function. *Thorax* 55: 271–276, 2000.
7. **Haberg SE, Stigum H, Nystad W, Nafstad P.** Effects of pre- and postnatal exposure to parental smoking on early childhood respiratory health. *Am J Epidemiol* 166: 679–686, 2007.
8. **Haberg SE, London SJ, Nafstad P, Nilsen RM, Ueland PM, Vollset SE, Nystad W.** Maternal folate levels in pregnancy and asthma in children at age 3 years. *J Allergy Clin Immunol* 127: 262–264, 2011.
9. **Hanrahan JP, Tager IB, Segal MR, Tosteson TD, Castile RG, Van VH, Weiss ST, Speizer FE.** The effect of maternal smoking during pregnancy on early infant lung function. *Am Rev Respir Dis* 145: 1129–1135, 1992.
10. **Hofhuis W, de Jongste JC, Merkus PJ.** Adverse health effects of prenatal and postnatal tobacco smoke exposure on children. *Arch Dis Child* 88: 1086–1090, 2003.
11. **Hollingsworth JW, Maruoka S, Boon K, Garantziotis S, Li Z, Tomfohr J, Bailey N, Potts EN, Whitehead G, Brass DM.** In utero supplementation with methyl donors enhances allergic airway disease in mice. *J Clin Invest* 118: 3462–3469, 2008.
12. **Joad JP, Ji C, Kott KS, Bric JM, Pinkerton KE.** In utero and postnatal effects of sidestream cigarette smoke exposure on lung function, hyperresponsiveness, and neuroendocrine cells in rats. *Toxicol Appl Pharmacol* 132: 63–71, 1995.
13. **Joad JP, Bric BM, Peake JL, Pinkerton KE.** Perinatal exposure to aged and diluted sidestream cigarette smoke produces airway hyperresponsiveness in older rats. *Toxicol Appl Pharmacol* 155: 253–260, 1999.
14. **Kaati G, Bygren LO, Edvinsson S.** Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period. *Eur J Hum Genet* 10: 682–688, 2002.
15. **Karadag A, Sakurai R, Wang Y, Guo P, Desai M, Ross MG, Torday JS, Rehan VK.** Effect of maternal food restriction on fetal rat lung lipid differentiation program. *Pediatr Pulmonol* 44: 635–644, 2009.
16. **Krebs M, Sakurai R, Torday JS, Rehan VK.** Evidence for in vivo nicotine-induced alveolar interstitial fibroblast-to-myofibroblast transdifferentiation. *Exp Lung Res* 36: 390–839, 2010.
17. **Leslie FM.** Multigenerational epigenetic effects of nicotine on lung function (Abstract). *BMC Med* 11: 27, 2013.
18. **Li YF, Langholz B, Salam MT, Gilliland FD.** Maternal and grandmaternal smoking patterns are associated with early childhood asthma. *Chest* 127: 1232–1241, 2005.
19. **Liu J, Naeem E, Tian J, Lombardi V, Kwong K, Akbari O, Torday JS, Rehan VK.** Sex-specific perinatal nicotine-induced asthma in rat offspring. *Am J Respir Cell Mol Biol* 48: 53–62, 2013.
20. **Liu J, Sakurai R, O'Roark EM, Kenyon NJ, Torday JS, Rehan VK.** PPARgamma agonist rosiglitazone prevents perinatal nicotine exposure-induced asthma in rat offspring. *Am J Physiol Lung Cell Mol Physiol* 300: L710–L717, 2011.
21. **Lumey LH.** Decreased birthweights in infants after maternal in utero exposure to the Dutch famine of 1944–1945. *Paediatr Perinat Epidemiol* 6: 240–253, 1992.
22. **Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK.** Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. *PLoS One* 8: e55387, 2013.
23. **March of Dimes.** *Alcohol and Drugs* 2010.
24. **Maritz GS, Woolward KM, du Toit G.** Maternal nicotine exposure during pregnancy and development of emphysema-like damage in the offspring. *S Afr Med J* 83: 195–198, 1993.
25. **Masoli M, Fabian D, Holt S, Beasley R.** The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* 59: 469–478, 2004.
26. **Mattes J, Collison A, Plank M, Phipps S, Foster PS.** Antagonism of microRNA-126 suppresses the effector function of TH2 cells and the development of allergic airways disease. *Proc Natl Acad Sci USA* 106: 18704–13809, 2009.
27. **Matthews SG, Phillips DI.** Minireview: transgenerational inheritance of the stress response: a new frontier in stress research. *Endocrinology* 151: 7–13, 2010.
28. **Miller RL, Garfinkel R, Horton M, Camann D, Perera FP, Whyatt RM, Kinney PL.** Polycyclic aromatic hydrocarbons, environmental tobacco smoke, and respiratory symptoms in an inner-city birth cohort. *Chest* 126: 1071–1078, 2004.

29. **Miller RL, Ho SM.** Environmental epigenetics and asthma: current concepts and call for studies. *Am J Respir Crit Care Med* 177: 567–573, 2008.
30. **Moshammer H, Hoek G, Luttmann-Gibson H, Neuberger MA, Antova T, Gehring U, Hruba F, Pattenden S, Rudnai P, Slachtova H.** Parental smoking and lung function in children: an international study. *Am J Respir Crit Care Med* 173: 1255–1263, 2006.
31. **Niedzwiecki M, Zhu H, Corson L, Grunig G, Factor PH, Chu S, Jiang H, Miller RL.** Prenatal exposure to allergen, DNA methylation, and allergy in grandoffspring mice. *Allergy* 67: 904–910, 2012.
32. **Pembrey ME, Bygren LO, Kaati G, Edvinsson S, Northstone K, Sjostrom M, Golding J.** Sex-specific, male-line transgenerational responses in humans. *Eur J Hum Genet* 14: 159–166, 2006.
33. **Pentinat T, Ramon-Krauel M, Cebria J, Diaz R, Jimenez-Chillaron JC.** Transgenerational inheritance of glucose intolerance in a mouse model of neonatal overnutrition. *Endocrinology* 151: 5617–5623, 2010.
34. **Rehan VK, Asotra K, Torday JS.** The effects of smoking on the developing lung: insights from a biologic model for lung development, homeostasis, and repair. *Lung* 187: 281–289, 2009.
35. **Rehan VK, Liu J, Naeem E, Tian J, Sakurai R, Kwong K, Akbari O, Torday JS.** Perinatal nicotine exposure induces asthma in second generation offspring (Abstract). *BMC Med* 10: 129, 2012.
36. **Roseboom TJ, Watson ED.** The next generation of disease risk: are the effects of prenatal nutrition transmitted across generations? Evidence from animal and human studies. *Placenta* 33, *Suppl* 2: e40–e44, 2012.
37. **Sakurai R, Li Y, Torday JS, Rehan VK.** Curcumin augments lung maturation, preventing neonatal lung injury by inhibiting TGF-β signaling. *Am J Physiol Lung Cell Mol Physiol* 301: L721–L730, 2011.
39. **Skinner MK.** Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability. *Epigenetics* 6: 838–842, 2011.
40. **Torday JS.** The sex difference in type II cell surfactant synthesis originates in the fibroblast in vitro. *Exp Lung Res* 7: 187–194, 1984.
41. **Torday JS, VK.** Rehan: Stretch-stimulated surfactant phospholipid synthesis is mediated by the coordinated paracrine actions of parathyroid hormone-related protein, and leptin. *Am J Physiol Lung Cell Mol Physiol* 283: L130–L135, 2002.
42. **Torday JS, Torres E, Rehan VK.** The role of fibroblast transdifferentiation in lung epithelial cell proliferation, differentiation, and repair in vitro. *Pediatr Pathol Mol Med* 22: 189–207, 2003.
43. **Torday JS, Rehan VK.** Developmental cell/molecular biologic approach to the etiology and treatment of bronchopulmonary dysplasia. *Pediatr Res* 62: 2–7, 2007.
44. **Whitrow MJ, Moore VM, Rumbold AR, Davies MJ.** Effect of supplemental folic acid in pregnancy on childhood asthma: a prospective birth cohort study. *Am J Epidemiol* 170: 1486–1493, 2009.
45. **Wigle DT, Arbuckle TE, Turner MC, Berube A, Yang Q, Liu S, Krewski D.** Epidemiologic evidence of relationships between reproductive and child health outcomes and environmental chemical contaminants. *J Toxicol Environ Health B Crit Rev* 11: 373–517, 2008.
46. **Youngson NA, Whitelaw E.** Transgenerational epigenetic effects. *Annu Rev Genomics Hum Genet* 9: 233–257, 2008.
47. **Zeybel M, Hardy T, Wong YK, Mathers JC, Fox CR, Gackowska A, Oakley F, Burt AD, Wilson CL, Anstee QM.** Multigenerational epigenetic adaptation of the hepatic wound-healing response. *Nat Med* 18: 1369–1377, 2012.





OPEN

Paternal nicotine exposure defines different behavior in subsequent generation via hyper-methylation of *mmu-miR-15b*

Received: 10 October 2016
Accepted: 5 July 2017
Published online: 04 August 2017

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The neurobehavioral effects of paternal smoking and nicotine use have not been widely reported. In the present study, nicotine exposure induced depression in the paternal generation, but reduced depression and promoted hyperactivity in F1 offspring. While this intergenerational effect was not passed down to the F2 generation. Further studies revealed that nicotine induced the down-regulation of mmu-miR-15b expression due to hyper-methylation in the CpG island shore region of *mmu-miR-15b* in both the spermatozoa of F0 mice and the brains of F1 mice. As the target gene of mmu-miR-15b, *Wnt4* expression was elevated in the thalamus of F1 mice due to the inheritance of DNA methylation patterns from the paternal generation. Furthermore, the increased expression of *Wnt4* elevated the phosphorylation level of its downstream protein GSK-3 through the canonical WNT4 pathway which involved in the behavioral alterations observed in F1 mice. Moreover, *in vivo* stereotaxic brain injections were used to induce the overexpression of mmu-miR-15b and WNT4 and confirm the neurobehavioral effects *in vitro*. The behavioral phenotype of the F1 mice resulting from paternal nicotine exposure could be attenuated by viral manipulation of mmu-miR-15b in the thalamus.

Tobacco smoking is one of the most severe public health issues worldwide[1] and it has recently been associated with mental disorders, such as depression, attention-defic t hyperactivity disorder (ADHD), among others. In the smoking population, the lifetime prevalence of major depressive disorder (MDD) appears to be markedly higher, with rates as high as 53% (compared to 6% to 10% in non-smokers)[2]. From another perspective, patients suffering from depression are much more likely to smoke tobacco than those who do not have an underlying depressive disorder[3,4]. These epidemiological data strongly support the high comorbidity of smoking with depressive symptoms because depression increases the risk of smoking and smoking increases the risk of depression. As one of the most hazardous substances in tobacco, nicotine plays a pivotal role in tobacco addiction[5]. As a consequence, there is an urgent need for additional research concerning the effects of nicotine on the nervous system because of the increasing popularity of E-cigarettes[6,7]. There were very limited studies in both humans and animals have indicated that nicotine treatment affects several aspects of emotionality, but the exact effects remain controversial[8,9]. Most mental disorders, including MDD and ADHD, are considered complex familial aggregative or heritable traits[10]. Therefore, it is worth considering whether the neuropsychiatric effects of smoking or nicotine exposure can be passed down to future generations. Most studies have focused on the effects of prenatal smoking or nicotine exposure on the mental health of the progeny[11–16]. ADHD is a developmental neuropsychiatric disorder,

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whose pathogenesis may be influenced by multiple environmental factors, including smoking and alcohol, and chemicals such as lead and polychlorinated biphenyls[17]. A limited number of studies have reported that paternal smoking influences the health of the next generation[18], but the effects on the mental health of off pring have not been widely reported. However, because of the high comorbidity of paternal smoking with maternal passive smoking, the intergenerational effects of paternal smoking *per se* is difficult to observe in the population; consequently, the establishment of a paternal smoking animal model is necessary and important.

Epigenetic studies on neuropsychiatric disorders have shown that environmental factors are also critical and that the genetic make-up of an individual contributes to heritability and disease risk. Theoretically, inherited non-genetic changes might represent evolutionary responses that enable rapid adaptation to environmental variation. Interestingly, a previous study reported that people traumatized during the genocide of the Khmer Rouge tended to have children who suffered from anxiety and depression[19]; this effect might be attributable to small non-coding RNAs (sncRNAs) in sperm, which can transmit acquired traits to progeny[20]. Other epigenetic modifi ations, such as DNA methylation and histone modifi ation, could also contribute to the passing of non-genetic changes to future generations[21–23]. These fi dings provide potential mechanisms for the intergenerational effects of paternal smoking or nicotine exposure. However, despite these advances, the current understanding of the father-to-off pring transmission of nicotine-induced behavioral changes remains unclear.

In the present study, we present an animal model of paternal smoking and nicotine exposure. In this model, sexually mature mice are exposed daily to tobacco smoke or intraperitoneal injections of nicotine. Exposure occurs throughout spermatogenesis to determine whether tobacco smoke or nicotine induce filial generational behavioral alterations. Th ough these efforts, we hope to characterize the molecular mechanisms underlying this putative intergenerational transmission process.

Results

Tobacco smoke exposure induced behavioral alternations in the treated mice and their progeny. F0 C57 male mice were subjected to tobacco smoke administration for 5 weeks to mimic long-term heavy smokers followed by behavioral tests to assess the neurobehavioral status of the mice. The tobacco smoke-treated mice (F0-smo) exhibited signifi antly longer immobility times (Fig. 1A, $P = 0.001$) in the forced swim test and reduced sucrose preferences (Fig. 1B, $P = 0.028$) compared with the non-smoking controls (F0-nos). In the open fi ld test, F0-smo mice moved signifi antly shorter distances (Fig. 1F, $P = 0.037$). These results indicated that the F0-smo mice in this animal model exhibited depression and a hypo-activity phenotype. In other behavioral tests, including elevated plus maze (Fig. 1C), novel object recognition (Fig. 1D), social chamber (Fig. 1E) and open fi ld tests (Fig. 1G–J), no signifi ant differences between the two groups were detected.

The F1-nos and F1-smo off pring were obtained, and subsequently, animal behavioral tests were conducted. In the forced swim test, the total time of immobility was signifi antly reduced in the paternal tobacco smoke-treated group compared with the controls (Fig. 1K, $P = 0.001$). In the social chamber test, F1-smo mice exhibited a greater length of time in the social chamber (Fig. 1O, $P = 0.042$). In the open fi ld test, the total distance moved (Fig. 1P, $p = 0.009$) and total vertical time (Fig. 1R, $p = 0.001$) of the F1-smo group were signifi antly elevated compared with the F1-nos group, while other parameters, including the total time of stereotypic behavior (Fig. 1Q), the total time in the central zone (Fig. 1S) and jump count (Fig. 1T), were not signifi antly different between the two groups. No signifi ant differences between the two groups were observed in the sucrose preference (Fig. 1L), elevated plus maze test (Fig. 1M) or novel object recognition test (Fig. 1N). These results suggest that tobacco smoke exposure might induce a depressive phenotype in the F0 generation, resulting in hyperactivity and activated social behavior in the F1 generation.

Nicotine exposure induced behavioral alterations in the treated mice and their progeny similar to that induced by tobacco smoke. After administration of this compound for 5 weeks, the neurobehavioral status of the mice was evaluated. The nicotine-treated mice (F0-nic) exhibited signifi antly longer immobility times (Fig. 2A, $P = 0.003$) in the forced swim test and a reduction in sucrose preference (Fig. 2B, $P = 0.030$) relative to controls (F0-con). These results suggested that the F0 mice in this model exhibited a depression-like phenotype. In the other behavioral tests (Fig. 2C–J, no signifi ant differences between the two groups were detected.

The F1-con and F1-nic offspring were obtained, and animal behavioral tests were conducted at 8–10 weeks of age. In the forced swim test, the total time of immobility was signifi antly shortened in the paternal nicotine-treated group compared with the controls (Fig. 2K, $P = 0.001$). In the open fi ld test, the total distance moved (Fig. 2P, $p = 0.037$), total time of stereotypic behavior (Fig. 2Q, $p = 0.007$) and total vertical time (Fig. 2R, $p = 0.019$) of the F1-nic group were signifi antly elevated compared with the F1-con group, while other parameters (Fig. 2S,T) were not signifi antly different. Additionally, no signifi ant differences between the two groups were observed in other behavioral tests (Fig. 2L–O). These results suggested that paternal nicotine exposure led to decreased levels of depression-like behavior and increased activity in the F1 off pring. Thus, the behavioral tests revealed that paternal nicotine exposure might induce a depressive phenotype in the F0 generation, while attenuating depression and inducing hyperactivity in the F1 generation.

Nicotine elevated the level of Wnt4 mRNA in the mouse spermatozoa. To acquire the gene transcription profiles of the sperm from the control and nicotine-treated F0 mice, murine sperm were purified (SFig. 1A), followed by the whole transcriptome sequencing, and the reads were mapped and analyzed. Figure 2A displays a heat map of the genes in the sperm differentially expressed resulting from nicotine treatment. The NGS results were subsequently validated using real-time PCR, which confi med these alterations (SFig. 1B). The discrepancies correlated with the neurobehavioral differences between the two groups were intensively scrutinized. Additional bioinformatic analyses revealed that the nicotine-induced alterations primarily involved the enrichment of the expression of genes in the Wnt signaling pathway (Fig. 3B); the key genes *Wnt4*, *Fzd9*, *Dvl2* and

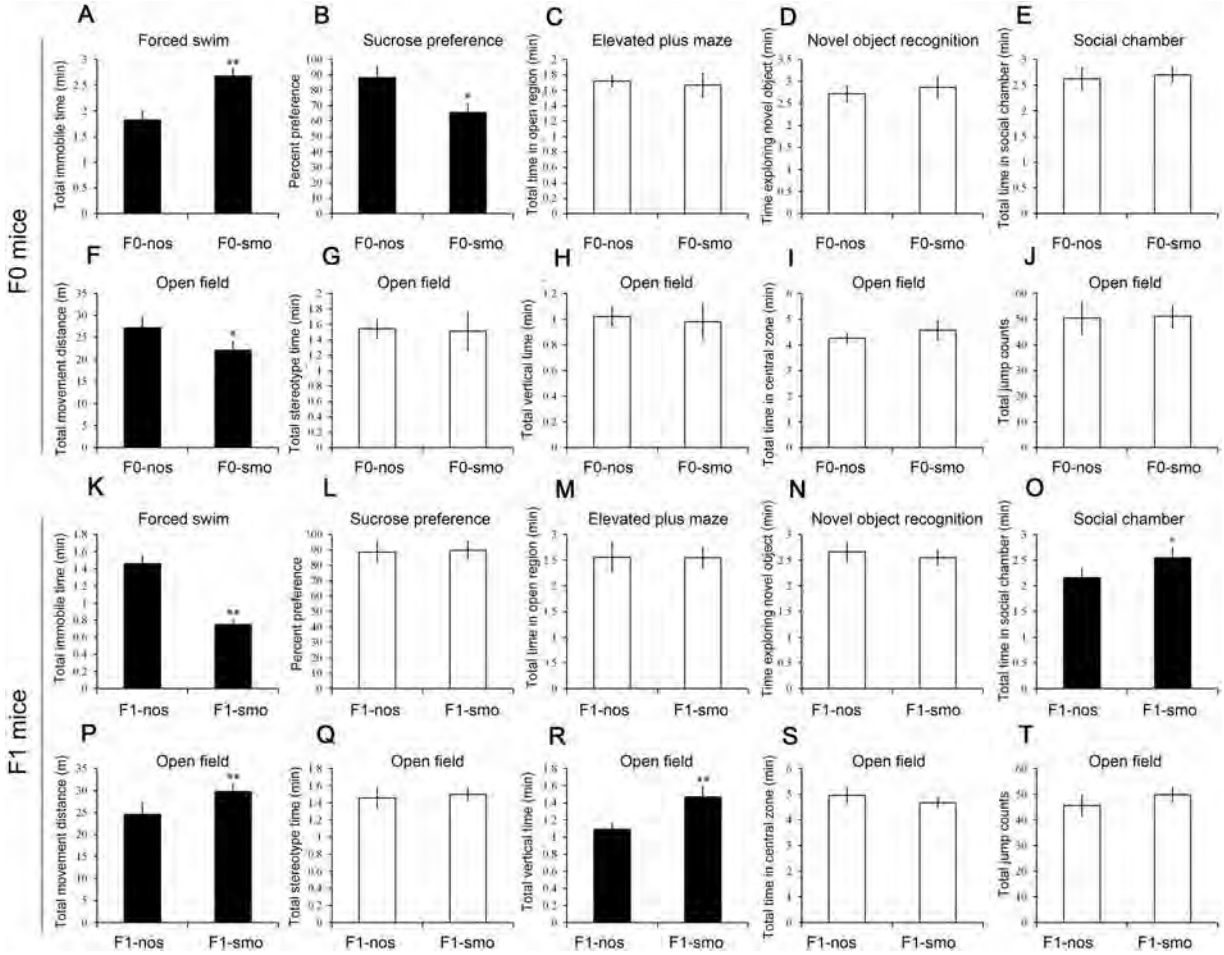

Figure 1. Behavioral tests of F0 and F1 mice of the tobacco smoke-treated and control groups (n = 20 for each group). (**A**) Forced swim tests of F0 mice from the tobacco smoke-treated and control groups. The histogram shows the total immobile times of the tested mice. (**B**) Sucrose preference test of F0 mice from the nicotine-treated and control groups. (**C**) Elevated plus maze test of F0 mice from the treated and control groups. (**D**) Novel object recognition test of F0 mice from the treated and control groups. (**E**) Social chamber test of F0 mice from the treated and control groups. (**F**–**J**) Open-fi ld test of F0 mice from the treated and control groups. (**K**) Forced swim test of F1 mice from the paternal tobacco smoke-exposure and control groups. (**L**) Sucrose preference test of F1 mice from the paternal tobacco smoke-exposure and control groups. (**M**) Elevated plus maze test of F1 mice from the paternal tobacco smoke-exposure and control groups. (**N**) Novel object recognition test of F1 mice from the paternal tobacco smoke-exposure and control group. (**O**) Social chamber test of F1 mice from the paternal tobacco smoke-exposure and control groups. (**P**–**T**) Open-fi ld test results for F1 mice from the paternal tobacco smoke-exposure and control groups.

Gsk3 were all signifi antly up-regulated in nicotine-treated mice (Fig. 3C). In a previous study[24], we demonstrated that only Wnt4 mRNA, but not WNT4 protein, could be detected in murine spermatozoa; accordingly, we only investigated the protein expression of Wnt family members in the brain tissue of F1 mice. The results revealed that only Wnt4 was signifi antly elevated in the F1-nic compared with the F1-con group, and other Wnt members (Fig. 2D and E) were not affected.

Paternal nicotine exposure induced activation of the Wnt4 pathway in F1 mouse brain. As the mRNA sequencing results suggested that the Wnt4 pathway might play an important role in the intergenerational effects of nicotine, the expression of two key proteins in Wnt4 signaling, WNT4 and Dishevelled 2 (DVL2) were measured using Western blot analysis (Fig. 3A) in the brain and testis tissues of F0 and F1 mice. The corresponding histograms for WNT4 (Fig. 3B) and DVL2 (Fig. 4C) revealed that nicotine elevated the expression of both proteins in the F0 testis (WNT4: $P = 0.036$, DVL2: $P = 0.001$) and the F1-brains (WNT4: $P = 0.003$, DVL2: $P = 0.012$), but not in the F0-brains. In the F1 mouse brains, the activation of Wnt4 signaling, which is closely associated with neurobehavioral status, was further investigated. Within the Wnt4 pathway, Wnt4 induces the normal inactivation of glycogen synthase kinase 3 (GSK3), evidenced by the stabilization of β-catenin and the stimulation of downstream gene transcription. Western-blot analysis (Fig. 4D and E) indicated that the expression of GSK-3α and -3β was signifi antly down-regulated, while phosphorylation (p-GSK3α/β) was elevated after

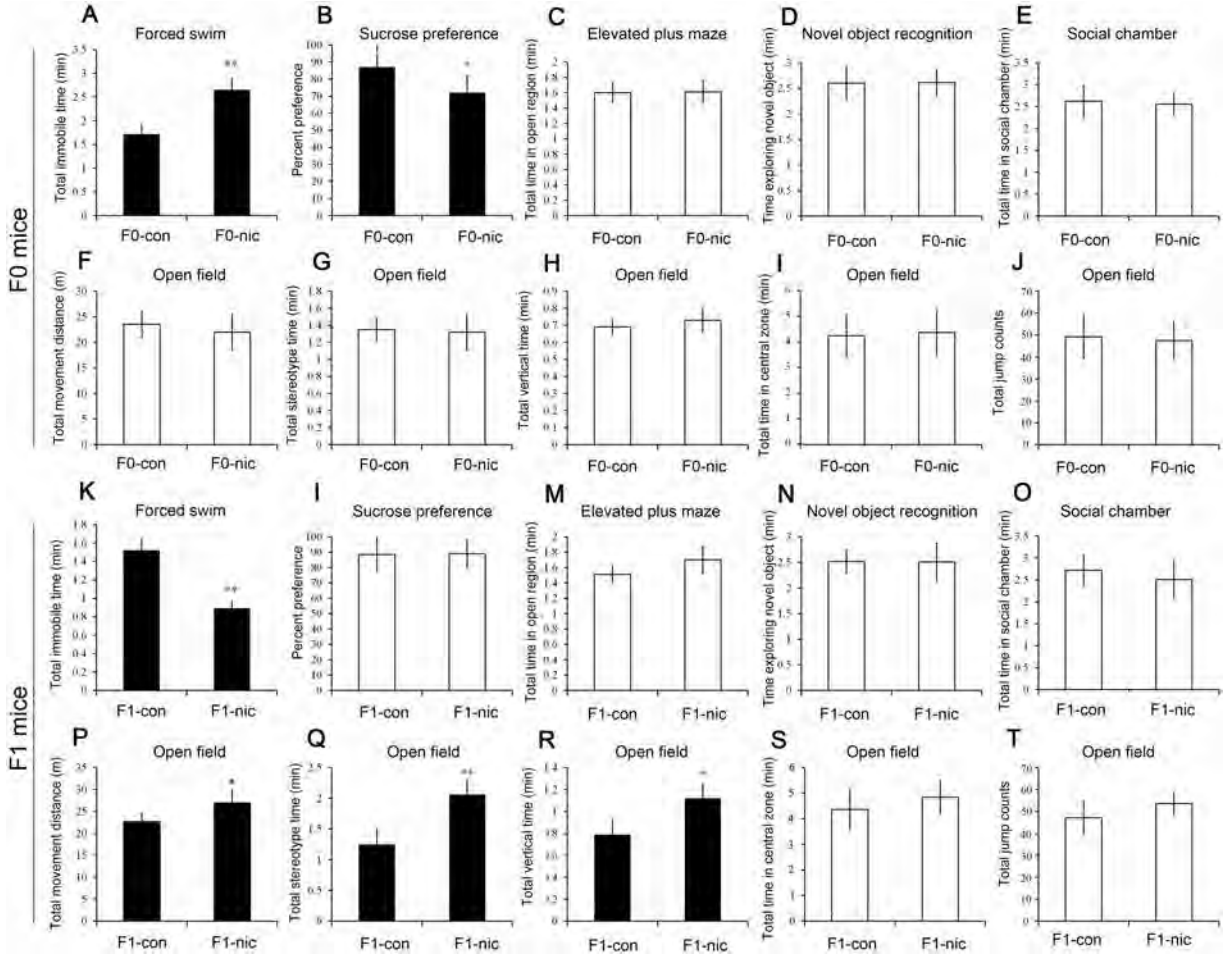

Figure 2. Behavioral tests of F0 and F1 mice of the nicotine-treated and control groups (n = 20 for each group). (**A**) Forced swim tests of F0 mice from the nicotine-treated and control groups. (**B**) Sucrose preference test of F0 mice from the nicotine-treated and control groups. (**C**) Elevated plus maze test of F0 mice from the nicotine-treated and control groups. (**D**) Novel object recognition test of F0 mice from the nicotine-treated and control groups. (**E**) Social chamber test of F0 mice from the nicotine-treated and control groups. (**F–J**) Open-fi ld test of F0 mice from the nicotine-treated and control groups. (**K**) Forced swim test of F1 mice from the paternal nicotine-exposure and control groups. (**L**) Sucrose preference test of F1 mice from the paternal nicotine-exposure and control groups. (**M**) Elevated plus maze test of F1 mice from the paternal nicotine-exposure and control groups. (**N**) Novel object recognition test of F1 mice from the paternal nicotine-exposure and control group. (**O**) Social chamber test of F1 mice from the paternal nicotine-exposure and control groups. (**P–T**) Open-fi ld test results for F1 mice from the paternal-nicotine exposure and control groups.

nicotine treatment. For the mRNA level, *Wnt4* were elevated in both the sperm of F0-nic mice and the brain of F1-nic mice while the *Dvl2* was only found elevated in brain of F1-nic mice (SFig. 2A and B).

We determined the distribution of WNT4 in the F1-nic mouse brains via immunofluorescence. As shown in Fig. 4F, WNT4 was primarily expressed in the thalamus (TH), and the expression level of WNT4 in the brains of F1-nic mice was greater than that seen in the controls. Because it is a downstream target of activated Wnt4, the distribution of DVL2 was also assessed. DVL2 was highly expressed in the hippocampal formation (HPF). Figure 4G illustrates that DVL2 was signifi antly expressed in the HPF, particularly in the CA3 region, but not in the dentate gyrus (DG) (SFig. 2C), in the F1-nic brain. Paternal nicotine exposure was also found to elevate DVL2 expression in the CA3 region of F1-nic mice. Thus, we speculated that in the F1 off pring following paternal nicotine exposure, the expression of WNT4 would be up-regulated in the TH, and the Wnt4 pathway would be activated in the HPF via a paracrine manner.

Mmu-miR-15b down-regulated the translation of Wnt4 mRNA. Initially, we investigated the DNA methylation level in the promoter region of *Wnt4* in the TH of F1 mouse brains and the sperm of F0 mice. The promoter region of Wnt4 was enriched in CpGs (SFig. 3A); however, these sites were hypo-methylated, and no signifi ant differences were observed between the controls and the nicotine-treated samples (SFig. 3B and C). As

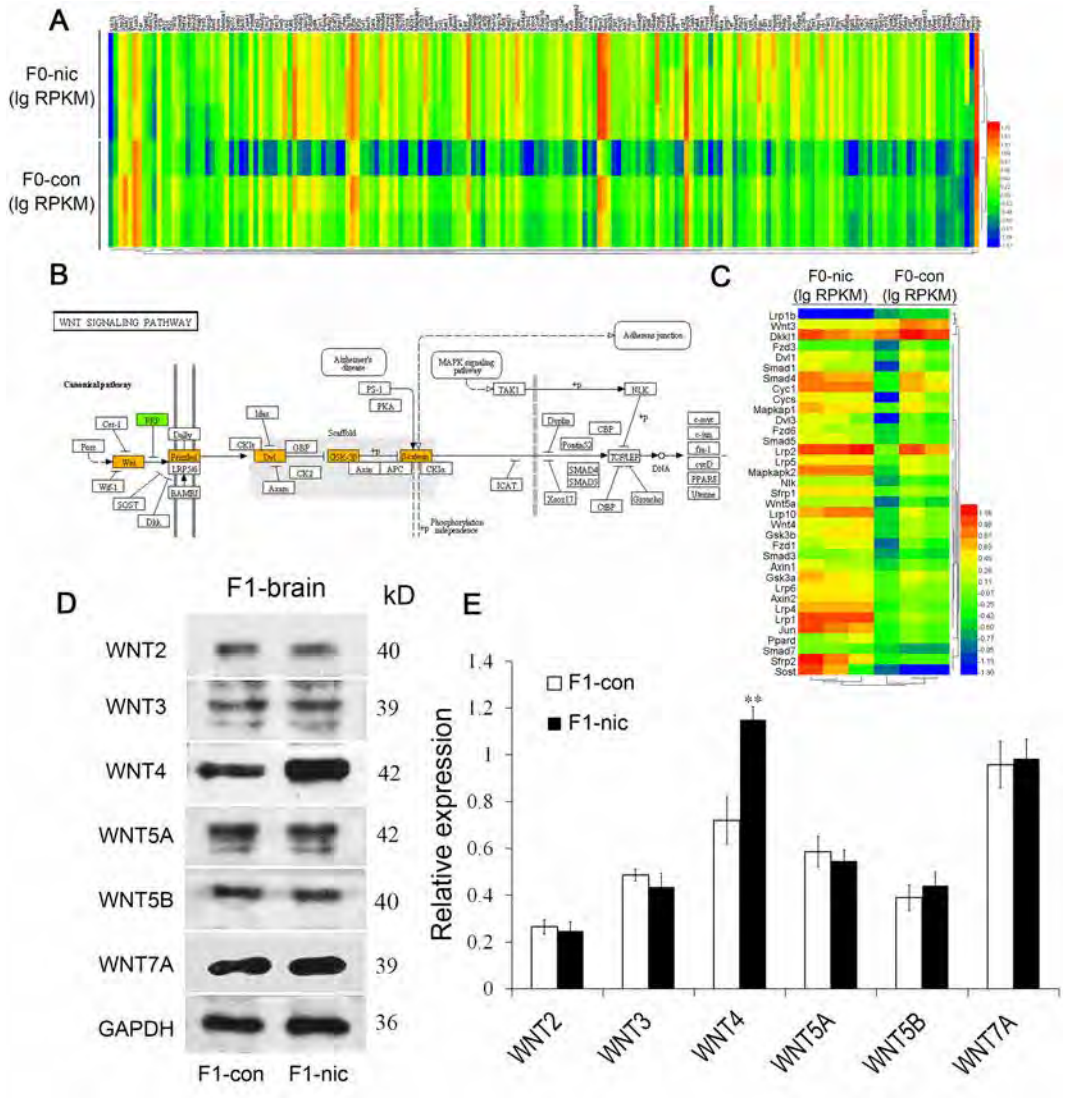

Figure 3. Transcriptomics analyses of the spermatozoa from nicotine-treated and control mice. (**A**) Heat map of Wnt/beta-catenin signaling-related mRNAs in the spermatozoa from nicotine-treated and control mice. The expression levels of mRNAs were normalized to lgRPKM prior to the transcriptomics analysis. (**B**) Schematic diagram of the canonical WNT signaling pathway (n = 3). (**C**) Heat map of key mRNAs in the Wnt signaling pathway in the spermatozoa of nicotine-treated and control mice. (**D**) Western blot results for WNT2, WNT3, WNT4, WNT5A, WNT5B and WNT7A from F1 brain tissue from the nicotine-treated and control groups. (**E**) A histogram of the western blot results for WNT2, WNT3, WNT4, WNT5A, WNT5B and WNT7A from F1 brain tissue (n = 3).

we did not detect any change in methylation state of the Wnt4 promoter, we decided to evaluate changes in miRNAs, another form of epigenetic regulation.

The TargetScan database revealed 7 miRNAs as the most likely candidates (Fig. 5A) that might regulate Wnt4 expression levels. Real-time PCR was performed to investigate the expression of mmu-miR-1907, mmu-miR-15a, mmu-miR-15b, mmu-miR-497, mmu-miR-16, mmu-miR-322 and mmu-miR-195 in the brain tissue of F1 mice from the two groups. The results indicated that only mmu-miR-15b was signifi antly down-regulated after nicotine exposure (Fig. 5B, P = 0.001). Moreover, we used PCR to analyze the expression of mmu-miR-15b in the spermatozoa of F0 mice and obtained results similar to those obtained from the F1 brains. Specifi ally, nicotine treatment signifi antly attenuated mmu-miR-15b expression (P = 0.028) in F0 mice spermatozoa (Fig. 5C). To determine whether mmu-miR-15b binds the 3′-UTR of Wnt4 mRNA and down-regulates Wnt4 expression, we cloned the 3′-UTR of Wnt4 into the psiCHECK-2 luciferase reporter vector, and a mutant of the 3′-UTR reporter with the mutation of 3 nucleotides was also constructed (Fig. 5D and E) and the dual-luciferase reporter assay was performed in HeLa cells. As shown in Fig. 5F, the expression of mmu-miR-15b signifi antly inhibited luciferase activity (P = 0.002), and the ability to inhibit reporter activity was lost in the mutant construct. We further transfected the mmu-miR-15b mimic into the TM3 cell line, which constitutively expresses WNT4. As shown in

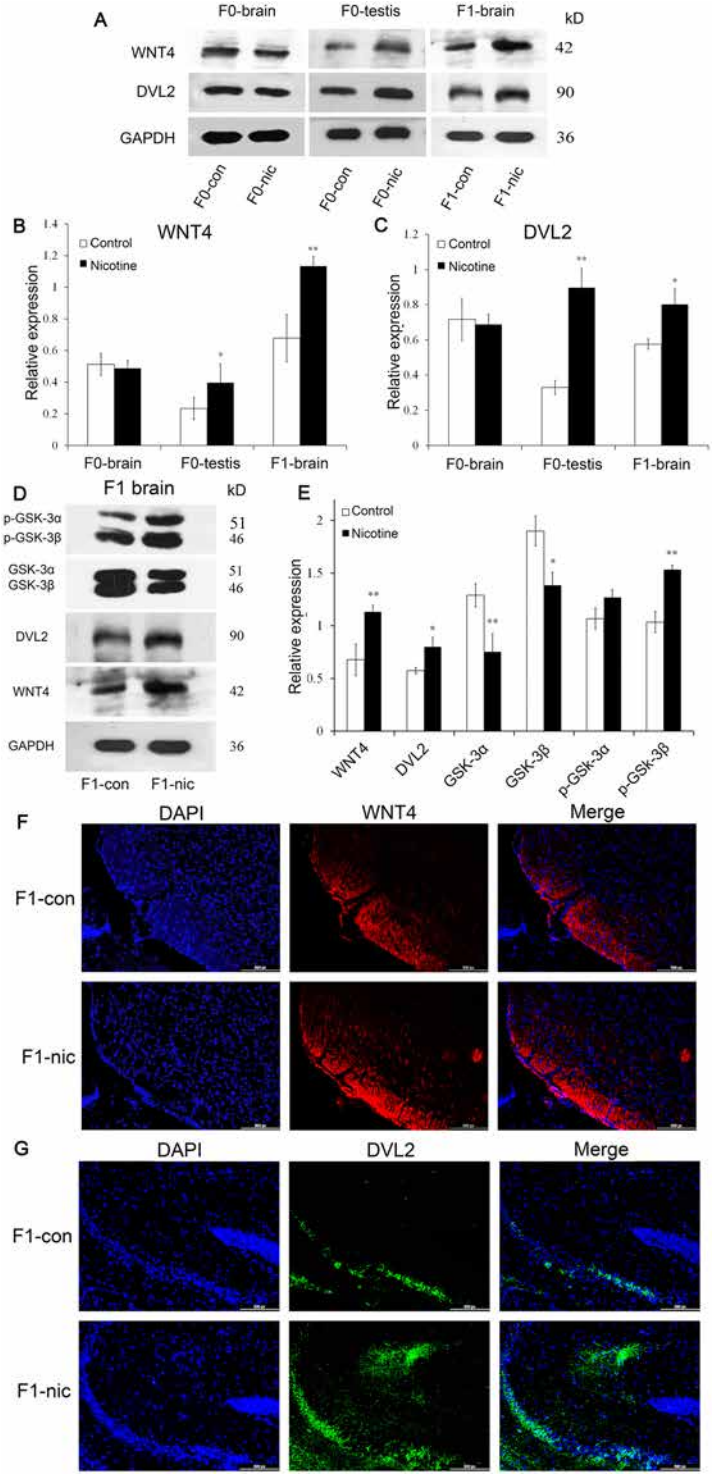

Figure 4. Expression levels and distribution of key proteins of Wnt4 signaling in different tissues. (**A**) Western blot results for WNT4 and DVL2 in F0-brain, F0-testis and F1-brain tissue of the two groups. (**B**) A histogram of the western blot results for WNT4 (n = 3). (**C**) A histogram of the western blot results for DVL2 (n = 3). (**D**) Western blot films of WNT4, DVL2 and GSK3 from F1-brain tissue from the nicotine-treated and control groups. (**E**) A histogram of the western blot results for WNT4, DVL2 and GSK3 in F1-brain tissue (n = 3). (**F**) Immunofluorescence staining for WNT4 (red) and DNA (blue) of brain samples from F1-con and F1-nic mice. (**G**) Immunofluorescence staining for DVL2 (green) and DNA (blue) in brain samples obtained from F1-con and F1-nic mice.

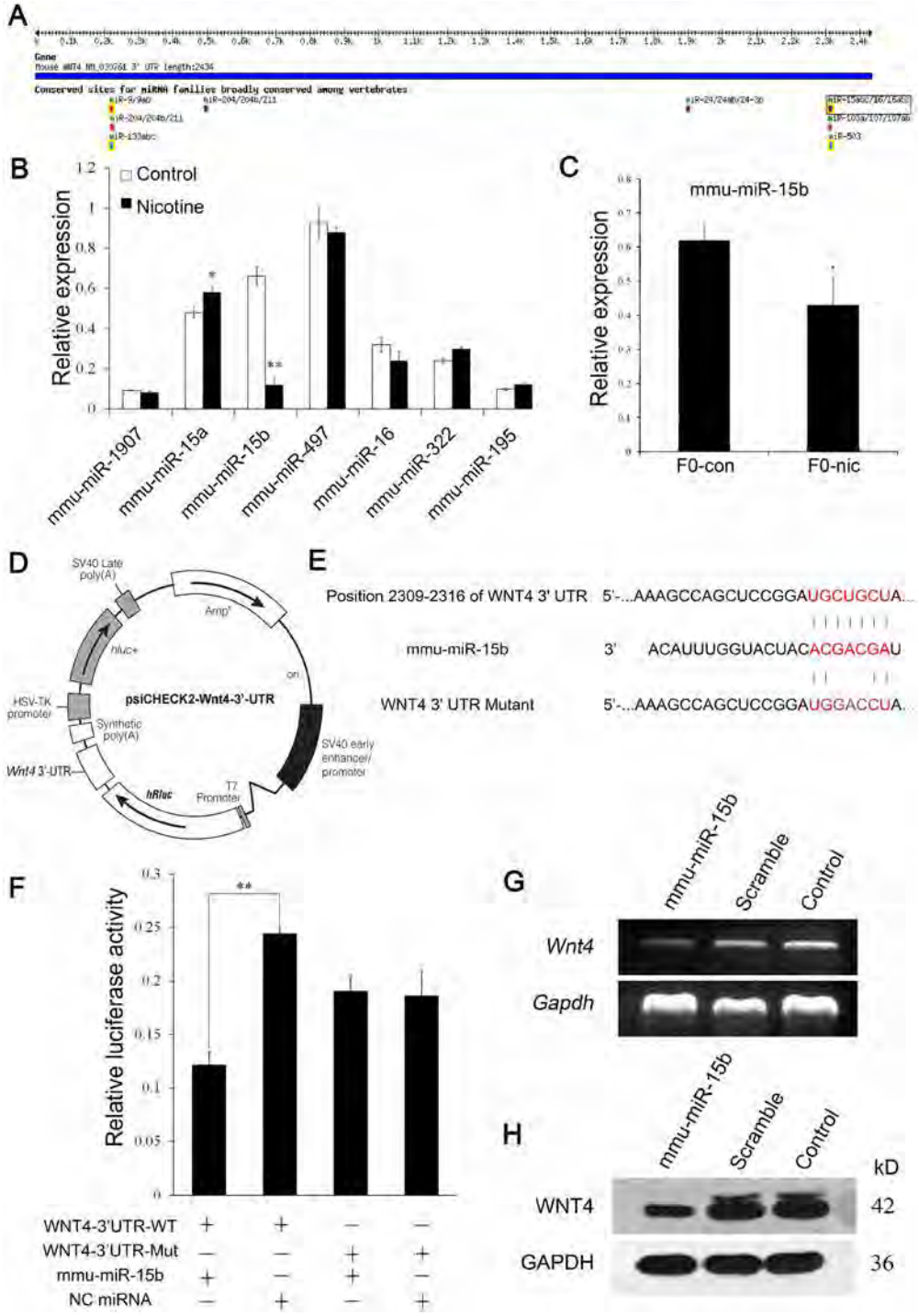

Figure 5. mmu-miR-15b down-regulates the translation of Wnt4 mRNA. (**A**) Diagrammatic sketch of the 3′-UTR region of the murine *Wnt4* gene. The miR-16 microRNA family, including miR-15a, miR-15b, miR-16, miR-1907, miR-497, miR-322 and miR-185, were predicted to bind the *Wnt4* 3′-UTR region. (**B**) Real-time PCR results for the expression levels of these miRNAs in F1-brain tissue. (**C**) Real-time PCR analysis of the mmu-miR-15b in the spermatozoa of F0 mice (n = 4). (**D**) The plasmid profile of the constructed dual-luciferase reporter vector psiCHECK-2-Wnt4 3′-UTR. (**E**) The nucleotide sequences of mmu-miR-15b and the complementary region of the *Wnt4* 3′-UTR. The mutant *Wnt4* 3′-UTR was constructed via point mutations of 3 nucleotides in the middle of the complementary region. The red letters illustrate the sequence alignment of mmu-miR-15b, the Wnt4 3′-UTR and the mutant Wnt4 3′-UTR. (**F**) Luciferase activity refl cting WNT4 expression in HeLa cells was suppressed by mmu-miR-15b, but not NC miRNA. The inhibitory action of mmu-miR-15b was abrogated when the target sites of the *Wnt4* 3′-UTR were mutated (n = 5). (**G**) Western blot results for WNT4 following transfection of mmu-miR-15b into TM3 cells. (**H**) Reverse-transcription PCR results for *Wnt4* after the transfection of mmu-miR-15b into TM3 cells.

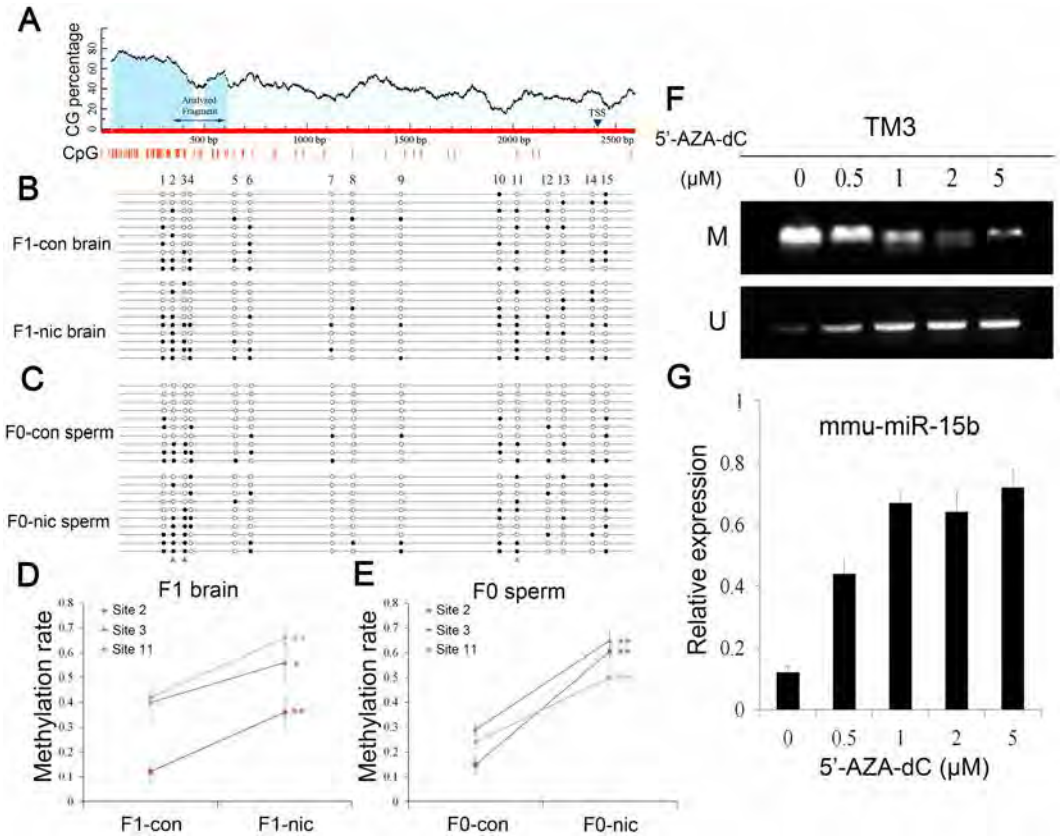

Figure 6. DNA methylation analysis within the CpG shore region of the murine *mmu-miR-15b* gene. (**A**) The predicted CpG island and bisulfite-sequencing PCR primers were designed using the Methprimer tool. The CpG islands are indicated with gray backgrounds, and the BSP primers flanked a 303-bp PCR product upstream of the transcription start position (TTS) of *mmu-miR-15b*. (**B**) The DNA methylation status of the CpG shore region of *mmu-miR-15b* in the thalami of F1 mice from the control and paternal nicotine-exposure groups. Each spot indicates one methylation site (CpG); the black spots indicate methylated cytosines, and the white spots indicate unmethylated cytosines. (**C**) The DNA methylation status of the CpG shore region of *mmu-miR-15b* in the spermatozoa of F0 mice from the control and nicotine-treated groups. (**D**) A histogram of the DNA methylation ratios of the analyzed CpG sites 2, 3 and 11 in the thalami of F1 mice from the control and paternal nicotine-exposure groups (n = 3). (**E**) A histogram of the DNA methylation ratios of the analyzed CpG sites 2, 3 and 11 in the spermatozoa of F0 mice from the control and nicotine-treated groups (n = 3). (**F**) Verifi ation of epigenetic regulation in the mouse TM3 cell line. The panels show the MSP results for the primers designed for the methylated and unmethylated sites in TM3 cells treated with a gradient of 5′-aza-dC concentrations. (**G**) The histogram shows the results of real-time PCR analyses of mmu-miR-15b expression levels in TM3 cells treated with 5′-aza-dC (n = 3).

Fig. 5G and H, the Wnt4 expression in TM3 cells transfected with the mmu-miR-15b mimic was attenuated at both the mRNA and protein levels.

Nicotine induced DNA hyper-methylation in the CpG island shore region of *mmu-miR-15b* in F1 mouse brains and F0 mouse spermatozoa. To further examine whether an epigenetic element of *mmu-miR-15b* is involved in paternal imprinting, we performed DNA methylation analyses of the promoter region of the *mmu-miR-15b* gene. The promoter region of *mmu-miR-15b* lacks CGIs; however, the enrichment of CpG sites in the CGI shore region (Fig. 6A) indicated that *mmu-miR-15b* might be an epigenetically modifi d gene. Furthermore, we investigated the DNA methylation patterns of the CGIs using primers flanking a 303-bp fragment for bisulfite sequencing analysis. Figure 6B and C illustrate the DNA methylation patterns of the F1-brain and F0-sperm, respectively. The BSP results indicated that the DNA methylation levels of *mmu-miR-15b* in the CGI shore region were signifi antly elevated at sites 2, 3 and 11 in the F1-brain and F0-sperm after nicotine treatment (Fig. 6D and E). Next, a DNA methylation verifi ation test was conducted using TM3 cells and the results confi med that mmu-miR-15b expression is elevated by 5′-AZA-dC, which attenuated the methylation levels in the CGI shore region of *mmu-miR-15b* (Fig. 6F and G).

Overexpression of mmu-miR-15b recapitulates a depression-like phenotype. Furthermore, we assessed the neurobehavioral effects of mmu-miR-15b and WNT4 *in vivo* through the viral manipulation of mmu-miR-15b and WNT4 in the TH region. A diagram of the construct and representative images of the lentivirus-mediated *Wnt4* gene transfer in the murine thalamus are shown in Fig. 7A and B, and those for

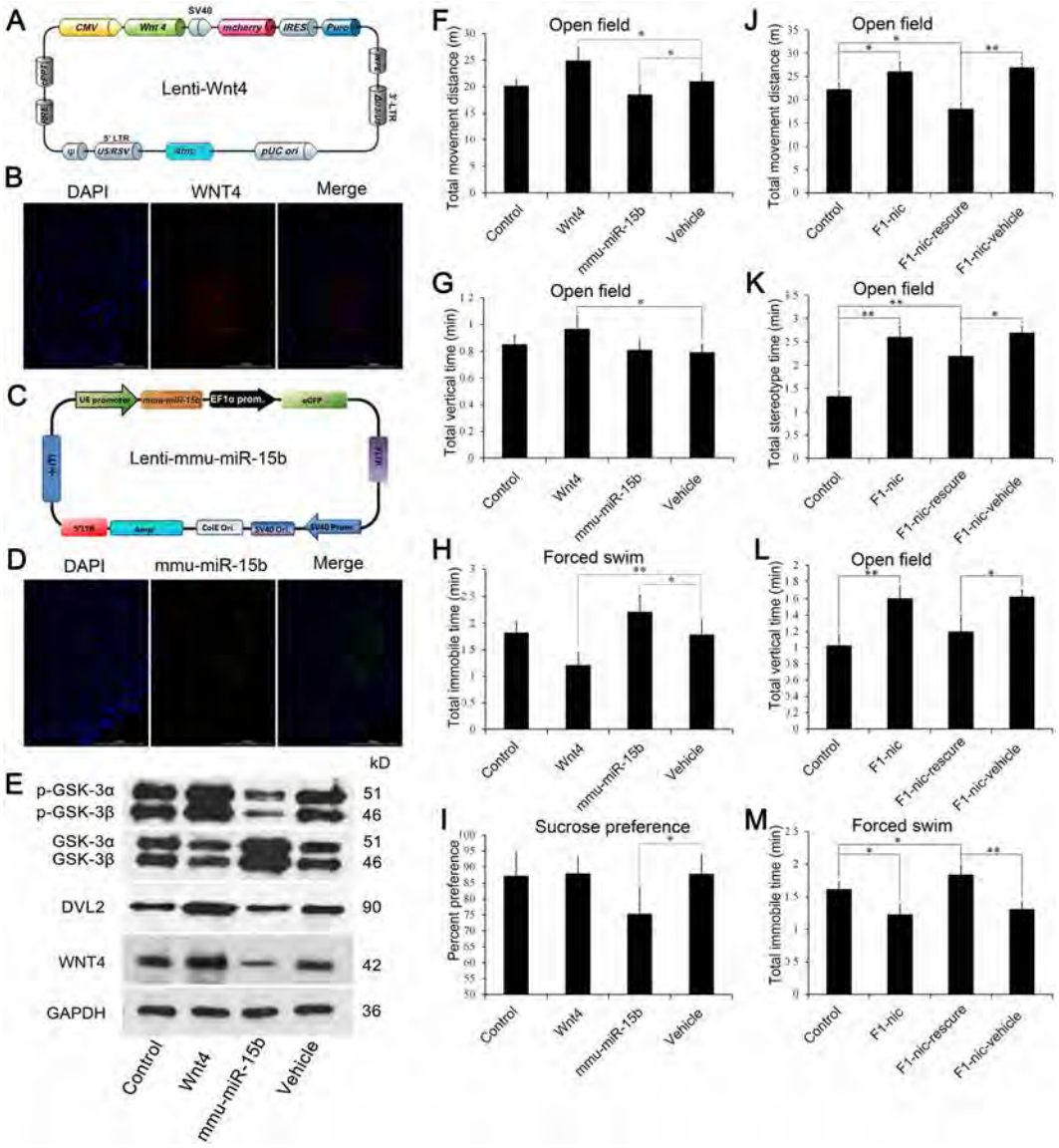

Figure 7. Viral manipulation of mmu-miR-15b and WNT4 in the TH region using brain stereotaxic injection confi med the neurobehavioral effects of *mmu-miR-15b* and *Wnt4 in vivo*. (**A**) Schematic presentation of the constructed Lenti-Wnt4 vector. (**B**) Representative example of lentivirus-mediated gene transfer in the mouse TH region. The red signal in the fluorescence micrograph shows the viral manipulation of WNT4. (**C**) Schematic presentation of the constructed Lenti-mmu-miR-15b vector. (**D**) Representation of lentivirus-mediated gene transfer in the mouse TH region. The green signal in the fluorescence micrograph shows the viral manipulation of mmu-miR-15b. (**E**) Western blot results for the key proteins in Wnt4 cascade in the TH region of mice that were subjected to the viral manipulated of WNT4 and mmu-miR-15b. (**F–I**) Animal behavioral results of mice subjected to lentivirus-mediated gene transfer in the TH region. The histogram shows the total movement distance of the mice (n = 8–12 for each group). (**J–M**) Animal behavioral results of F1-nic mice subjected to lentivirus-mediated overexpression of mmu-miR-15b in the TH region. The F1-nic-15b group were subjected to the viral manipulation of mmu-miR-15b, while the F1-nic-vehicle group received injections of blank lentivirus (n = 8–12 for each group).

mmu-miR-15b are shown in Fig. 7C and D. The expression level of the key proteins in Wnt4 cascade in the thalamus of the mice receiving intra-thalamic injections were measured via Western blot analysis and the results validated the effici cy of the viral manipulation (Fig. 7E). C57/BL6J mice then received bilateral intra-thalamic injections of Lenti-Wnt4 or Lenti-mmu-miR-15b, separately, with the empty vector used as a control. These mice were subsequently subjected to behavioral tests to evaluate their neurobehavioral status. In the open-fi ld test, the total distance moved (Fig. 7F, $P = 0.018$) and total vertical time (Fig. 7G, $P = 0.029$) were significantly elevated in the Lenti-Wnt4-injected group, and the total distance moved was reduced in the Lenti-mmu-miR-15b group (Fig. 7F, $P = 0.034$) compared with the vehicle-only group. In the forced swim test (Fig. 7H), the total time of immobility was significantly lower ($P = 0.008$) in the Lenti-Wnt4-injected group and higher in the

Lenti-mmu-miR-15b group (P = 0.036) compared with the vehicle-only group. In the sucrose preference test, the sucrose preference percentage was signifi antly decreased in the Lenti-mmu-miR-15b transfected group compared with the vehicle group (Fig. 7I, P = 0.019). The other behavioral tests revealed no signifi ant differences between the experimental and vehicle groups (SFig. 4A–F).

To determine whether the behavioral phenotype of F1-nic mice could be attenuated by viral manipulation, the F1-nic mice were also received lentiviral-mediated overexpression of mmu-miR-15b in the thalamus. After 2 weeks of recovery, these mice were subjected to behavioral tests to evaluate their neurobehavioral status. In the open-fi ld test, the overexpression of mmu-miR-15b in the thalamus down-regulated the total distance moved (Fig. 7J, P = 0.002), total time performing stereotypic behaviors (Fig. 7K, P = 0.023) and total vertical time (Fig. 7L, P = 0.025) in F1-nic mice. After the viral manipulation of mmu-miR-15b, the experimental group exhibited a signifi antly lower total distance moved (Fig. 7J, P = 0.028) compared with control mice, while the total time of stereotyped behaviors did not return to the control level (Fig. 7K, P = 0.008). For the total vertical time, the experimental group showed no difference compared with controls (Fig. 7L). In the forced swim test, the overexpression of mmu-miR-15b signifi antly elevated the total time of immobility in F1-nic mice (Fig. 7M, P = 0.004), and this parameter was higher compared with control mice (P = 0.039). Based on the behavioral tests, the viral manipulation of mmu-miR-15b in the thalamus of F1-nic mice partially attenuated the behavioral phenotypes resulting from paternal nicotine exposure.

The neurobehavioral phenotype of F1-nic mice was not passed down to the F2 generation. To observe whether the neurobehavioral phenotype of F1-nic mice is passed down to the F2 generation, the F2-nic and F2-con mice were generated using cross-fostering method as previously described. The animal behavioral tests were performed and the results showed no significant differences between the two groups (SFig. 5A–J). Subsequently, the potential molecular transmission through the paternal line mentioned above was analyzed. Supplementary Fig. 5K shows the western blot films of WNT4 and DVL2 in the brain tissue of F2 mice from the nicotine-treated and control groups, and there was no signifi ant difference between the two groups (SFig. 5L). Real-time PCR analysis of mmu-miR-15b was also performed, and no significant difference was observed between the two groups in the spermatozoa of F1 mice and in the brain tissue of F2 mice. Furthermore, the DNA methylation status of the CpG island shore region of *mmu-miR-15b* was also measured in the spermatozoa of F1 mice and the TH region in F2 mice, and the methylation rates of 3 specific CpG sites showed no signifi ant differences between the two groups (SFig. 5N).

Discussion

In present study, daily tobacco smoke exposure induced a depression-like phenotype in the F0 generation, resulting in hyperactivity and activated social behavior in the F1 generation. While daily moderate nicotine exposure replicated the depression-like phenotype in the F0 generation and attenuated the depressive level, resulting in hyperactivity in the F1 generation. Remarkably, the murine model of nicotine exposure recapitulated most behavioral phenotypes in the tobacco exposure model, consistent with human epidemiological studies. It is worth noting that in addition to molecular mechanisms, parental information can also be passed to the offspring via cultural or social inheritance systems[25]. However, maternally provided social inheritance is unlikely in our paternal effect system because of the cross-fostering method. As a consequence, the molecular etiology underlying the behavioral alterations observed in the F1-nic mice are most likely inherited from the epigenetic information in the sperm of the F0 mice. Therefore, the intergenerational effects transmitted via the male germline were the focus of the present study. From the gene transcription profiling of the sperm of F0 mice, we observed that the nicotine-induced genes were primarily enriched in the Wnt4 signaling pathway. The disruption of Wnt signaling adversely affects brain development and has been associated with the pathophysiology of several neurological disorders[26]. Recent studies have suggested important roles for the Wnt signaling pathway in bipolar disorder (BP)[27] and MDD[28]. Multiple components of the Wnt signaling pathway have been implicated in neurogenesis and neurological anomalies, including Frizzled receptors, Dvl and GSK3β. Dvl and β-catenin play critical roles in axon differentiation and dendritic arborization[29]. Moreover, The expression level of GSK3β has been associated with anxiety-like and depression-like behaviors[30,31]. In addition to expression levels, GSK3α and GSK3β are regulated by inhibitory phosphorylation on Ser21-GSK3α and Ser9-GSK3β. The inhibitory control of GSK3 is crucial for the normal function of neurons[32]. Deficie cies in the signaling pathways that normally maintain the inhibition of GSK3 could induce the up-regulation of GSK3 activity, thereby promoting susceptibility to depression[33]. In the present study, paternal nicotine exposure induced the up-regulation of the Wnt4 pathway in the thalamus of the F1 mouse brain. WNT4-induced inhibitory phosphorylation of GSK3 might represent the molecular etiology of the behavioral alterations discussed above.

Multiple epigenetic mechanisms have also been implicated in the etiology of multiple psychiatric disorders[34]. The results of the present study indicated that the over-expression of mmu-miR-15b in the mouse brain induced hypoactivity and depression-like behavior. Herein, we validated *Wnt4* as a novel target gene of mmu-miR-15b using a dual-luciferase assay. Mmu-miR-15b is highly conserved relative to human miR-15b. Increases in the cortical expression of miR-15b have previously been associated with schizophrenia[35] but the underlying mechanism remains unknown. At the molecular level, miR-15b plays an important role in cell proliferation and apoptosis by targeting the mRNA of *CCND1* (Cyclin D1) and *BCL2* (*B-cell CLL/lymphoma 2*)[36]. Based on these reported target genes, the other effects of attenuated mmu-miR-15b levels in the mouse brain resulting from paternal nicotine exposure require further study, particularly in terms of neurogenesis. Besides miRNAs, DNA methylation is the most widely studied epigenetic mechanism and tobacco smoking is perhaps the best-studied environmental factor affecting DNA methylation. There are robust correlations between prenatal maternal smoking and DNA methylation patterns in the umbilical cord, placenta, and off pring[37]. In the present study, we focused only the DNA methylation and miRNAs in sperm, but our data do not rule out the possibility of inheritance through other

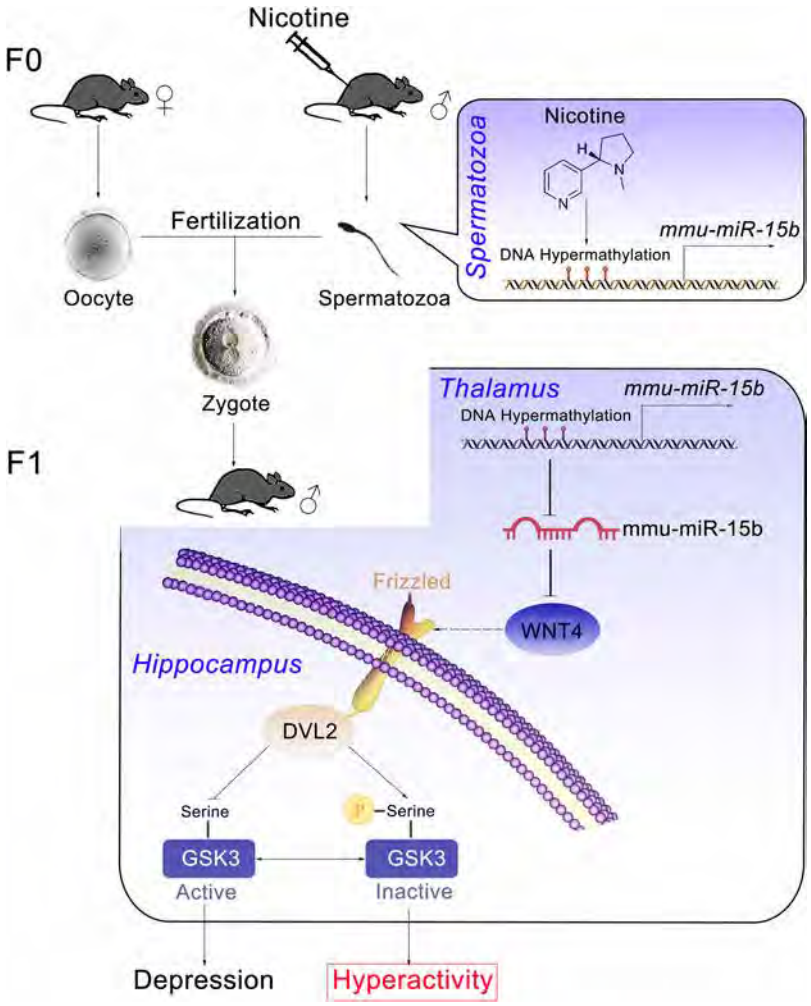

Figure 8. Diagrammatic sketch of the mechanisms underlying paternal nicotine exposure-induced behavioral alterations in the F1 generation.

epigenetic mechanisms. The expression level of mmu-mmiR-15b was regulated by DNA methylation in the CGI shore region, and alterations in DNA methylation patterns resulting from nicotine exposure were observed in the spermatozoa of F0 mice and the brains of F1 mice. Additionally, RNA-dependent processes in sperm also contribute to the transmission of acquired traits in mammals[20], but the precise mechanism remains unknown. We proposed that the alterations of the sncRNA levels in sperm might only be markers or side effects of other epigenetic modifi ations. In the present study, nicotine elevated the DNA methylation level in the CGI shore of *mmu-miR-15b* in the sperm of F0 mice. Tissue- and disease-specific differentially methylated DNA regions occur more frequently within CGI shores than within CGIs[38]. Such epigenetic modifi ations are subsequently passed to the next generation and imprint the brain, leading to the induction of neurobiological abnormities. Moreover, the intergenerational effects mediated by mmu-miR-15b were not passed down to the F2 generation. Th s phenomenon might suggest the reprogramming of DNA methylation during spermatogenesis in F1-nic mice. Evolutionarily, the reprogramming of epigenetic modifi ations can be considered as a protective mechanism that insulates further generations from the intergenerational effects of environment substances. The precise molecular mechanism underlying the epigenetic regulation of the CGI shore of mmu-miR-15b during spermatogenesis and embryonic development remains elusive.

As summarized in Fig. 8, the present study provides solid evidence regarding the mechanisms underlying paternal nicotine exposure-induced behavioral alterations in F1 generations. Nicotine exposure induces epigenetic mmu-miR-15b downregulation due to CGI shore hyper-methylation in murine spermatozoa, and these epigenetic modifi ations may mediate the intergenerational inheritance of nicotine-induced neuropsychological disorder. Reduced mmu-miR-15b further elevates the expression of its target gene Wnt4 in the thalamus of F1 brains, followed by the activation of the Wnt4 pathway. The canonical Wnt4 pathway induces inhibitory GSK3 phosphorylation, ultimately inducing hyperactivity and alleviating depression in the off pring.

Methods

Animals and Nicotine Treatments. The animal experiments in this study were approved by the bioethics committee of Shanghai Jiao Tong University and all animal studies were performed in accordance with the China

State Food and Drug Administration (SFDA) guidelines. Forty 6-week-old male C57BL/6 J mice (acquired from Shanghai SLAC Laboratory Animal Co. Ltd.) were randomly divided into control and nicotine-treated groups containing 20 mice each. The nicotine-treated group (F0-nic) received total 0.2 mg/100 g free-base nicotine via intraperitoneal injection to mimic the blood plasma levels of nicotine in heavy smokers ($\geq$20 cigarettes/day)[39]. The mice received nicotine treatment 4 times per day (q.q.h. in daytime); thus, a lower dose (0.05 mg/100 g) was received in each injection to avoid malaise or sickness. The control group (F0-con) received a daily equivalent of saline. After 5 weeks of treatment, the F0-con and F0-nic mice were mated with normal female C57BL/6 J mice to generate the F1 off pring, designated as the F1-con and F1-nic groups, respectively. The male mice continued to receive nicotine treatment during mating (5 days) until the copulation plugs were observed. Subsequently, the male mice were removed from dam after mating to prevent any exposure to the pups. In the absence of nicotine exposure, when F1 mice were sexually mature, 40 F1 mice from each of the F1-nic and F1-con groups were randomly selected to perform behavioral tests. The mice were group housed with free access to food and water and maintained on a 12:12 h light: dark cycle. For the tobacco smoke-treated mice (F0-smo), an apparatus was designed to expose mice to tobacco smoke, mimicking the exposure of heavy smokers[40]. Specifically, a 150 W vacuum pump was used to generate the suction to draw cigarette smoke into a 30 cm $\times$ 30 cm glass box. After the mice were placed in the box, the vacuum pump was concurrently turned on to draw cigarette smoke into the box. After depleting the cigarette, the vacuum pump was turned off and the mice continued to be exposed to the smoke for 1 h. On each occasion, 20 mice were exposed, and the mice were exposed twice a day at an interval of 1 h. The mice in the control group (F0-nos) were placed in a box under identical conditions, but no cigarette was lit. After 5 weeks of treatment, the F0-smo and F0-nos mice were also mated with normal female mice to generate F1 off pring (F1-smo and F1-nos) for further behavioral analysis. All the F1 mice were cross fostered according to the standard protocol.

Open field Test. In the open field task, each mouse was gently placed into the center of an open field (27.5 cm $\times$ 27.5 cm $\times$ 25 cm) and remained in the box for a period of 20 minutes. Exploration and arousal were measured as the number of grids crossed and reared using a photo beam activity system. The total movement distance, total stereotype time, total vertical time, time in the central zone and total jump counts were measured.

Elevated Plus Maze. The elevated plus maze tests were performed in an apparatus comprising four arms (5 cm $\times$ 5 cm $\times$ 30 cm): two oppositely positioned arms were closed with opaque walls, and the other two arms were open. All of the arms were interconnected via a 5 cm $\times$ 5 cm platform, and the apparatus was elevated at 60 cm above the ground. Individual mice were placed at the distal end of one open arm facing away and released. The total time in the open region was measured and analyzed.

Novel Object Recognition. During training, the mice were placed in the experimental box and exposed to two identical objects for 10 min. After 24 h, the mice were placed back into the same box for the NOR test. To avoid bias resulting from general object preference, the two objects (A and B) used during training and testing were counterbalanced, such that half of the mice in each group were trained with object A and tested with object B and half of the mice in each group were trained with object B and tested with object A. The times that the mice spent with old and novel objects were analyzed.

Social Chamber Test. The mice were placed in a clear acrylic box partitioned into three chambers. The corners of the two side chambers housed empty black wire-mesh cylinders. The mice were examined under two conditions. Under the fi st condition, the mice were placed in the center chamber. The partitions were removed, and the animal was permitted to freely explore the chambers. The times and frequencies the animals spent in each of the three chambers and around the cylinders were recorded. The mice were subsequently placed back in the center chamber after 10 min. In the second condition, an unfamiliar C57BL/6J mouse was placed in one cylinder. The test mice were subsequently permitted to explore the chamber and the times and frequencies spent in the three chambers and at the cylinder were recorded.

Forced Swim Test. The mice were forced to swim for a 5-min period in a vessel containing 40-cm-deep water maintained at 25 $\pm$ 1 °C. The time taken to reach the fi st fl ating state (i.e., an immobile state with only small limb movements to remain afl at) and the total time spent in an immobile state were measured and analyzed.

Sucrose Preference Test. The animals were administered two identical 50-ml bottles with sipper tubes containing water for 2 days. On the third day, the animals were provided with one bottle of drinking water, and the other bottle contained 1% sucrose. The amounts of water and sucrose consumed were measured each day over the next 4 days and recorded as the percentage of sucrose consumed.

RNA Sequencing and Subsequent Bioinformatics Analysis. After an adult male mouse was euthanized, the cauda of the epididymis was carefully isolated, and the spermatozoa were forced out after a nick was cut in the cauda epididymis. The isolated spermatozoa were suspended in human tube for subsequent treatments. The spermatozoa suspended in HTF were purifi d through the swim-up method in Earles balanced salt containing 10% fetal bovine serum and then washed with hypotonic solution containing 0.5% Triton X-100 and 0.1% SDS to lyse the remaining somatic cells and immature sperm. The samples for RNA extraction were ultimately suspended in TRIzol reagent, and the total RNAs were extracted following the manufacturer's instructions. Approximately 20 μg of purifi d RNA submitted for sequencing. Following reverse transcription with random primers, the samples were segmented, and the adaptors were added. A TruSeq^{TM} DNA Sample Prep Kit-Set A (Illumina) was used to construct the library. The samples from 3 mice in each group were subsequently sequenced using an Illumina GAIIx Genome Analyzer with 100 cycles and paired-end sequencing. Using the fastx package, clean reads were

obtained from the raw data after filtering out the reads containing more than 5% unknown nucleotides and the low-quality reads. "TOPHAT" was used after the reads and junction reads were mapped to the reference sequence. The data concerning gene expression levels estimated from the RPKM values were obtained using "Cuffl ks." All of the data analysis protocols for the sequenced segments were performed according to commonly used methods that have previously been reported[41]. The Blast2GO comprehensive bioinformatics tool was used for functional annotation and ontology analyses of the gene and protein sequences and gene ontology analyses. Genomatix (http://www.genomatix.com) was used for functional analyses of the target genes. The pathway analyses were performed using the KEGG database (http://www.genome.jp/kegg). Web Gestalt (http://bioinfo.vanderbilt.edu/gotm) was used to analyze the phenotypes of the sequenced mRNAs.

Cell Culture and Treatments. The HeLa cell line was cultured in DMEM medium supplemented with 50 IU/ml penicillin, 50 IU/ml streptomycin and 10% FBS under 5% CO_2 at 37 °C, and the TM3 mouse Leydig cell line was cultured in DMEM-F12 medium. After the TM3 cells were cultured to approximately 90% confluence, 5-aza-2′-deoxycytidine (5-aza-dC, Sigma, Cat. No. A3656) was added to the culture medium at fi al concentrations of 0, 0.5, 1 and 2 μM. The cells were harvested using TRIzol (Life Technologies) following 48 h of treatment, and the total mRNA, genomic DNA and protein were obtained according to standard protocols.

Dual Luciferase Reporter Assay. Luciferase reporters (psiCHECK2-Wnt4-3′-UTR) were constructed by inserting the 3′- UTR fragment of the mouse Wnt4 gene into a psiCHECK2 reporter vector (Promega) using the NotI/XhoI sites. Mutations of the 3′-UTR were generated via the mutation of 3 nucleotides based on the wild-type (WT) construct psiCHECK2- Wnt4-3′-UTR using the Takara MutanBEST Kit (Takara). The mmu-miR-15b mimic was synthesized at Shanghai GenePharma. HeLa cells were cotransfected with a luciferase reporter vector and mmu-miR-15b mimic or scrambled miRNA using Lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activities were sequentially measured using dual-luciferase assays (Promega) at 24 h after transfection according to the manufacturer's instructions.

Western Blot Analysis. Purifi d protein samples were isolated using TRIzol reagent and subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis, the separated proteins were transferred onto PVDF membranes. The PVDF membranes were blocked for 1 h at room temperature and subsequently incubated with diluted primary antibodies at 4 °C overnight. After rinsing 3 times with TBST, the membranes were incubated with secondary antibody (goat anti-rabbit IgG-HRP, at 1:20000 in TBST; Maibio) for 1 h at room temperature. After rinsing, HRP was detected using the Millipore Immobilon Western Chemiluminescent HRP substrate, and the final blot was exposed to X-ray film. The bands were scanned and equilibrated to the protein concentrations of the samples. The following primary antibodies were used for western blotting: GAPDH rabbit mAb (Cell Signaling Technology, Cat No. 2118S), WNT4 rabbit mAb (Sigma, HPA011397), DVL2 rabbit mAb, (Proteintech, 12037-1-AP), GSK3 rabbit mAb (Cell Signaling Technology, 9369S), p-GSK3 rabbit mAb (Cell Signaling Technology, 9327S), and DNMT3A rabbit mAb (Abcam, ab23565).

Real-time PCR and Reverse Transcription PCR. Purified RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and subsequently reverse transcribed into cDNA with a One-Step PrimeScript miRNA cDNA Synthesis Kit (Takara, D350A). Quantitative real-time PCR was performed using Bestar real-time PCR Master Mix (SYBR Green; DBI Bioscience) and an ABI PRISM 7500 system (Applied Biosystems) according to the manufacturer's instructions. The primers for real-time PCR were designed via Primer-blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized at Invitrogen. The transcript levels of the target genes were normalized against the inner reference gene U6. The relative quantifi ations (RQs) were performed using the $2^{-\Delta(\Delta Ct)}$ method, where RQ or the fold-change is equal to $2^{-((\text{Mean } \Delta Ct \text{ Target}) - (\text{Mean } \Delta Ct \text{ Calibrator}))}$.

Immunofluorescence (IF). The PFA-fi ed brain tissue of the mice from the two groups were immersed and embedded in paraffin Subsequently, 5-μm sections were cut and mounted onto poly-L-lysine-coated glass slides. The sections were baked at 85 °C for 15 min and subsequently deparaffini d according to standard protocols. The rehydrated sections were washed, and 0.01 M citrate buffer (pH 6.0) was used for antigen retrieval in a pressure-cooker. After natural cooling in citrate buffer, the sections were treated with 1% Triton X-100 for 15 min and incubated with 5% BSA (in TBS, pH = 7.4) for 30 min, followed by rinsing with TBS (3 × 5 min) and overnight incubation with the primary antibodies at 4 °C. After washing with TBS, the sections were incubated with the indicated FITC-conjugated mouse anti-goat IgG (Proteintech Group, SA00003-2, 1:200 in TBS) at 37 °C for 1 h. After washing three times with TBS, the sections were mounted using DAPI mounting medium. The fluorescence images were recorded with a Leica DM2500 fluorescence microscope.

Bisulfite Sequencing (BSP) and Methylation-Specific PCR (MSP). Purifi d genomic DNA was isolated using the phenol-chloroform extraction method. Next, 1 μg of purifi d DNA was treated using the Methylamp DNA Modification Kit (Epigentek) according to the manufacturer's instructions. The eluted DNA (5 μl) was PCR amplified using *mmu-miR-15b*-specific bisulfate sequencing primers (15b-BSP-S: 5′-GAAGTTTGTGGAGATTTTTTGAG-3′; and 15b-BSP-A: 5′-AAAAAACAATCCAACAATAAAAAAT-3′). The PCR product (303 bp) was recovered, purifi d and cloned into the pMD19-T vector (Takara, Japan). The cloning vector was transformed into competent bacteria, and 20 clones from the nicotine-treated and control samples were sequenced. The sequencing results were analyzed using BiQ analyzer (http://biq-analyzer.bioinf.mpi-inf.mpg.de/). For the methylation-specific polymerase chain reactions (MSPs), genomic DNA was isolated from 5-aza-dC-treated TM3 cells and subsequently treated with sodium bisulfate. Total volumes of 2.5 μl of the treated DNA were amplifi d with two sets of MSP primers (15b-M-S: 5′-AGGAATTTTATGTAGTTTGTTTTAACG-3′ and 15b-M-A: 5′-ACACCTACACTCATATACATATACCCG-3′; 15b-U-S:

5′-AATTTTATGTAGTTTGTTTTAATGG-3′ and 15b-U-A: 5′-ACACCTACACTCATATACATATACCCAC-3′). For the BSP and MSP, EX-Taq HS (Takara, RR006A) and touchdown PCR with annealing temperatures of 63 °C to 58 °C were used to ensure the generation of more specific PCR products. The 291-bp PCR products were resolved using 2% agarose gel electrophoresis.

Mouse Brain Stereotaxic Injection. C57BL/6J mice were anesthetized with pentobarbital sodium (8.5 mg/100 g) and placed into a stereotaxic apparatus (David Kopf). The skin was incised along the midline, and the connective tissue was removed with 10% H_2O_2. Burr holes were drilled after the bregma was located. A Hamilton syringe needle was lowered, and the vectors were infused bilaterally at a rate of 0.3 μl per minute in total volumes of 1 μl. The coordinates of the needle tip as measured from bregma were as follows: antero-posterior (AP): −1.6 mm; medial-lateral (ML): ±1.0; and dorsal-ventral (DV): −3.2 mm. The needle remained in place for 3 min following the injection to limit suction of the vector up the needle track.

Statistical Analysis. All statistical analyses were performed with SPSS version 11. The independent experiments were performed at least in triplicate, and all values are expressed as the means ± SD. Student's t-tests were used to compare the results between the two groups. Signifi ant differences are indicated by "*" ($P < 0.05$), and extremely signifi ant differences are indicated by "**" ($P < 0.01$).

Data Availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

1. Organization, W. H. *WHO report on the global tobacco epidemic, 2013: enforcing bans on tobacco advertising, promotion and sponsorship*. (World Health Organization, 2013).
2. Hitsman, B., Borrelli, B., McChargue, D. E., Spring, B. & Niaura, R. History of depression and smoking cessation outcome: a meta-analysis. *Journal of consulting and clinical psychology* **71**, 657 (2003).
3. Breslau, N., Peterson, E. L., Schultz, L. R., Chilcoat, H. D. & Andreski, P. Major depression and stages of smoking: a longitudinal investigation. *Archives of general Psychiatry* **55**, 161–166 (1998).
4. Pérez-Stable, E. J., Marin, G., Marín, B. V. & Katz, M. H. Depressive symptoms and cigarette smoking among Latinos in San Francisco. *American Journal of Public Health* **80**, 1500–1502 (1990).
5. Benowitz, N. L. Nicotine addiction. *The New England journal of medicine* **362**, 2295 (2010).
6. Clearing the smoke. *Nature neuroscience* **17**, 1013, doi:10.1038/nn.3777 (2014).
7. E-cigarettes–aid to smoking cessation or smokescreen? *Lancet* **384**, 829, doi:10.1016/s0140-6736(14)61470-7 (2014).
8. SalÄn-Pascual, R. J., Rosas, M., Jimenez-Genchi, A. & Rivera-Meza, B. L. Antidepressant effect of transdermal nicotine patches in nonsmoking patients with major depression. *Journal of Clinical Psychiatry* (1996).
9. Semba, J., Mataki, C., Yamada, S., Nankai, M. & Toru, M. Antidepressantlike effects of chronic nicotine on learned helplessness paradigm in rats. *Biol Psychiatry* **43**, 389–391 (1998).
10. Whalley, K. Psychiatric disorders: a feat of epigenetic engineering. *Nat Rev Neurosci* **15**, 768–769, doi:10.1038/nrn3869 [doi] nrn3869 [pii] (2014).
11. Stroud, L. R. *et al.* Maternal smoking during pregnancy and neonatal behavior: a large-scale community study. *Pediatrics* **123**, e842–e848 (2009).
12. Zhu, J. L. *et al.* Parental smoking during pregnancy and ADHD in children: the Danish national birth cohort. *Pediatrics* **134**, e382–e388 (2014).
13. Neuman, R. J. *et al.* Prenatal smoking exposure and dopaminergic genotypes interact to cause a severe ADHD subtype. *Biological psychiatry* **61**, 1320–1328 (2007).
14. Button, T., Thapar, A. & McGuffin P. Relationship between antisocial behaviour, attention-defic t hyperactivity disorder and maternal prenatal smoking. *The British Journal of Psychiatry* **187**, 155–160 (2005).
15. Nigg, J. T. & Breslau, N. Prenatal smoking exposure, low birth weight, and disruptive behavior disorders. *Journal of the American Academy of Child & Adolescent Psychiatry* **46**, 362–369 (2007).
16. Wakschlag, L. S., Pickett, K. E., Kasza, K. E. & Loeber, R. Is prenatal smoking associated with a developmental pattern of conduct problems in young boys? *Journal of the American Academy of Child & Adolescent Psychiatry* **45**, 461–467 (2006).
17. Biederman, J. & Faraone, S. V. Attention-defic t hyperactivity disorder. *Lancet (London, England)* **366**, 237–248, doi:10.1016/s0140-6736(05)66915-2 (2005).
18. Soubry, A., Verbeke, G. & Hoyo, C. Do early paternal exposures to lifestyle factors such as smoking increase the risk of chronic diseases in the off pring? *European journal of human genetics: EJHG*. doi:10.1038/ejhg.2014.206 (2014).
19. Hughes, V. Sperm RNA carries marks of trauma. *Nature* **508**, 296–297 (2014).
20. Gapp, K. *et al.* Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nature neuroscience* (2014).
21. Anway, M. D., Cupp, A. S., Uzumcu, M. & Skinner, M. K. Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* **308**, 1466–1469 (2005).
22. Dias, B. G. & Ressler, K. J. Parental olfactory experience influences behavior and neural structure in subsequent generations. *Nature neuroscience* **17**, 89–96 (2014).
23. Vassoler, F. M., White, S. L., Schmidt, H. D., Sadri-Vakili, G. & Pierce, R. C. Epigenetic inheritance of a cocaine-resistance phenotype. *Nature neuroscience* **16**, 42–47 (2013).
24. Fang, P. *et al.* Estimated diversity of messenger RNAs in each murine spermatozoa and their potential function during early zygotic development. *Biology of reproduction, biolreprod.* **114**, 117788 (2014).
25. Meaney, M. J., Szyf, M. & Seckl, J. R. Epigenetic mechanisms of perinatal programming of hypothalamic-pituitary-adrenal function and health. *Trends in molecular medicine* **13**, 269–277, doi:10.1016/j.molmed.2007.05.003 (2007).
26. De Ferrari, G. & Moon, R. The ups and downs of Wnt signaling in prevalent neurological disorders. *Oncogene* **25**, 7545–7553 (2006).
27. Matigian, N. *et al.* Expression profiling in monozygotic twins discordant for bipolar disorder reveals dysregulation of the WNT signalling pathway. *Molecular psychiatry* **12**, 815–825, doi:10.1038/sj.mp.4001998 (2007).
28. Inkster, B. *et al.* Association of GSK3β polymorphisms with brain structural changes in major depressive disorder. *Archives of general psychiatry* **66**, 721–728 (2009).
29. Yu, X. & Malenka, R. C. β-catenin is critical for dendritic morphogenesis. *Nature neuroscience* **6**, 1169–1177 (2003).
30. O'Brien, W. T. *et al.* Glycogen synthase kinase-3β haploinsufficiency mimics the behavioral and molecular effects of lithium. *The Journal of neuroscience* **24**, 6791–6798 (2004).

31. Beaulieu, J.-M. *et al.* Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 5099–5104 (2004).
32. Li, X. *et al. In vivo* regulation of glycogen synthase kinase-3β (GSK3β) by serotonergic activity in mouse brain. *Neuropsychopharmacology: offi al publication of the American College of Neuropsychopharmacology* **29**, 1426 (2004).
33. Polter, A. *et al.* Deficie cy in the inhibitory serine-phosphorylation of glycogen synthase kinase-3 increases sensitivity to mood disturbances. *Neuropsychopharmacology* **35**, 1761–1774 (2010).
34. Tsankova, N., Renthal, W., Kumar, A. & Nestler, E. J. Epigenetic regulation in psychiatric disorders. *Nature Reviews Neuroscience* **8**, 355–367 (2007).
35. Beveridge, N., Gardiner, E., Carroll, A., Tooney, P. & Cairns, M. Schizophrenia is associated with an increase in cortical microRNA biogenesis. *Molecular psychiatry* **15**, 1176–1189 (2009).
36. Xia, L. *et al.* miR-15b and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. *International journal of cancer* **123**, 372–379 (2008).
37. Markunas, C. A. *et al.* Identifi ation of DNA Methylation Changes in Newborns Related to Maternal Smoking during Pregnancy. *Environ Health Perspect* (2014).
38. Doi, A. *et al.* Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nature genetics* **41**, 1350–1353, doi:10.1038/ng.471 (2009).
39. Matta, S. G. *et al.* Guidelines on nicotine dose selection for *in vivo* research. *Psychopharmacology* **190**, 269–319 (2007).
40. Zhu, Z. *et al.* The alteration of protein profile induced by cigarette smoking via oxidative stress in mice epididymis. *Th international journal of biochemistry & cell biology* **45**, 571–582, doi:10.1016/j.biocel.2012.12.007 (2013).
41. Mortazavi, A., Williams, B. A., McCue, K., Schaeffer, L. & Wold, B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* **5**, 621–628 (2008).

Acknowledgements

Th s work was supported by the 973 Program (2010CB529600), the National Nature Science Foundation of China (81121001), the Shanghai Leading Academic Discipline Project (B205) and the Shanghai Key Laboratory of Psychotic Disorders. The authors also thank Prof. Dr. Cary Lai at the Department of Psychological and Brain Sciences and Program in Neuroscience, Indiana University for his critical reading and revising of the manuscript.

Author Contributions

Z.D., L.W. and W.X. designed the study. J.D., Z.Z. and M.Z. conducted the experiments. J.D. and Z.W. prepared the manuscript. J.D., X.Z., D.Z. and D.N. acquired and analyzed the experimental data.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-07920-3

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Nicotine & Tobacco Research, 2017, 1149–1154
doi:10.1093/ntr/ntw320
Original investigation
Received June 6, 2016; Editorial Decision November 21, 2016; Accepted November 30, 2016

Original investigation

Low Nicotine Content Descriptors Reduce Perceived Health Risks and Positive Cigarette Ratings in Participants Using Very Low Nicotine Content Cigarettes

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Abstract

Introduction: Understanding how smokers perceive reduced nicotine content cigarettes will be important if the FDA and global regulatory agencies implement reduced nicotine product standards for cigarettes. Prior research has shown that some smokers incorrectly believe "light" cigarettes are less harmful than regular cigarettes. Similar misunderstandings of health risk could also apply to reduced nicotine cigarettes. To date, most studies of reduced nicotine cigarettes have blinded subjects to the nicotine content. Therefore, little is known about how smokers experience reduced nicotine content cigarettes when they are aware of the reduced content, and how use may be impacted.

Methods: The present study was a within-subjects experiment with 68 adult daily smokers who smoked two identical very low nicotine content Quest 3 (0.05 mg nicotine yield) cigarettes. Subjects were told that one cigarette contained "average" nicotine content, and the other contained "very low" nicotine content. After smoking each cigarette, subjects completed subjective measures about their smoking experience.

Results: Subjects rated the "very low" nicotine cigarette as less harmful to their health overall compared to the "average" nicotine cigarette; this effect held true for specific smoking-related diseases. Additionally, they rated the "very low" nicotine cigarette as having less desirable subjective effects than the "average" nicotine cigarette and predicted having greater interest in quitting smoking in the future if only the "very low" nicotine cigarette was available.

Conclusions: Explicit knowledge of very low nicotine content changes smokers' perceptions of very low nicotine content cigarettes, resulting in reduced predicted harm, subjective ratings and predicted future use.

Implications: Before a reduced nicotine product standard for cigarettes can be implemented, it is important to understand how product information impacts how smokers think about and experience very low nicotine content cigarettes. Prior research has shown that smokers incorrectly believed light cigarettes were less harmful products. As such, smokers may also misunderstand the health risks associated with smoking very low nicotine content cigarettes.

This study highlights the importance of smokers' perceptions of nicotine content in cigarettes on the perceived health risks and the subjective effects of smoking very low nicotine content cigarettes.

Introduction

One possible regulatory strategy for reducing the harm caused by cigarettes is to implement a product standard requiring the reduction of nicotine content to make cigarettes less addictive.[1–3] Theoretically, a reduced nicotine product standard could improve public health through various pathways, including: (1) decreasing youth uptake if fewer adolescents progress from experimentation to addiction; (2) increasing smoking cessation in current smokers; and (3) reducing smoke exposure in current smokers who are unable to quit.[2] Several previous studies have explored the effect of extended use of very low nicotine content (VLNC) cigarettes in current smokers, and their results generally support the notion that a nicotine reduction policy might decrease smoking. [4–10]

Despite the aforementioned possible positive outcomes of a reduced nicotine product standard, unintended negative consequences might arise, which should be closely evaluated. For example, nicotine reduction could change smokers' beliefs about the health risks associated with smoking. Previous double-blind studies have shown that smokers rate cigarettes with greatly reduced nicotine content as having fewer health risks than cigarettes with relatively normal nicotine content,[11] indicating that "actual" nicotine content influences risk perception (perhaps through some sensory experience, though this is not known for sure). Less is known about how knowledge of the reduced nicotine content might affect risk perception. One study investigating the effect of advertisement features on risk beliefs found that when smokers naïve to Quest brand cigarettes (previously commercially available in three reduced nicotine levels: 0.6 mg, 0.3 mg and 0.05 mg nicotine yield) viewed a Quest cigarette advertisement that emphasized nicotine-free smoking, respondents incorrectly believed the product would be less harmful than their own cigarettes.[12] Other studies have shown that some smokers have an inaccurate understanding about nicotine's impact on smoking-related diseases (eg, the belief that nicotine causes lung cancer).[12–15] If a new product standard was to be implemented, smokers would likely be aware of the nicotine reduction and some may incorrectly reason that VLNC cigarettes have significantly fewer direct health consequences, which could negatively impact use. Indeed, research on "light" cigarettes suggests that smokers perceive them as less harmful.[16–18] However, since both nicotine and tar yields are lower in "light" cigarettes, it is unclear which factor is responsible for these misconceptions.

Previous research has established that nicotine content expectancies influence numerous subjective effects of smoking. For example, nicotine content expectancies significantly influence craving reduction, mood, wakefulness, calmness, concentration, satisfaction from smoking, and hunger reduction.[19–23] Other studies have found significant influences of nicotine content expectancies for other nicotine delivery products such as sprays and inhalers on satisfaction and craving relief.[24–26] These studies suggest the amount of nicotine that smokers believe is in a product can supersede their experience of receiving a different nicotine amount even at relatively high doses. These beliefs about nicotine content may influence the subjective experience of smoking and the perception of risk, which likely influences smoking behavior.[27]

The present study investigated the effect of a very low nicotine content expectancy on health risk perceptions and subjective effects of smoking cigarettes with actual low nicotine content. It was predicted that the "very low" nicotine cigarette would be perceived as less risky for contributing to various smoking-related diseases and rated as less subjectively desirable than the "average" nicotine cigarette (though both cigarettes were actually identical in nicotine content). Additionally, we explored whether the expectations of low nicotine content were associated with interest in quitting in the future.

Methods

Participants

Daily smokers aged 18 and older who smoked at least 10 cigarettes per day for the past year were recruited from the Pittsburgh community. An expired carbon monoxide level of at least 8 ppm (or urine cotinine level > 100 ng/m) was required for study enrollment. Exclusion criteria included significant medical changes in the previous week, currently seeking treatment to quit smoking, alcohol intoxication at the time of the visit, and pregnancy/breastfeeding.

Procedures

Following an initial phone screen, all eligible volunteers providing informed consent and completed a battery of baseline questionnaires. To standardize time since last cigarette, participants smoked four puffs of their usual brand cigarette through a smoking topography device and subsequently answered questions about their usual brand smoking experience.

Next, participants smoked two identical study cigarettes that differed only in the description of nicotine content (as described below). Order of nicotine content expectancy (ie, "average" nicotine cigarette or "very low" nicotine cigarette) was randomly assigned and stratified by gender. The cigarettes were separated by 45 minutes to avoid satiation. To assess possible changes in smoking behavior, the cigarettes were smoked through handheld smoking topography devices (CReSS Micro, Borgwaldt, KC). Both study cigarettes were 0.05 mg nicotine yield (Quest 3) and were matched to the participant's menthol preference. The Quest logo on the cigarette was obscured by permanent marker, so that participants remained blind to the brand. Staff was blind to the order of the expectancy conditions for each subject. After the research assistant left the room, the cigarette description for each condition appeared on the computer screen for 30 seconds and a recorded voiceover read the text aloud to ensure attention to the information. The text was as follows:

"The next cigarette that you will be smoking contains a very low/average nicotine level, compared to most cigarettes available in the United States. First, you will smoke as much or as little of this cigarette as you would like to smoke. Then, you will be asked to answer some questions about your opinions of the product."

The next slide instructed the participant to take the corresponding study cigarette from a large manila envelope (inside were two smaller envelopes labeled "very low nicotine cigarette" and "average nicotine cigarette"). Single cigarettes, rather than whole cigarette

packs, were in the smaller envelopes to maintain the product blind. Participants were instructed not to discuss the nicotine content of each cigarette with the research assistant and to put the materials away before the staff returned. Next, participants were told to place the cigarette into the puff topography device, light it, and smoke as much as desired. Cigarette rating measures were completed in reference to the study cigarette just smoked. This process was repeated for both study cigarettes.

Assessments

Demographic and smoking variables included age, gender, race, cigarettes per day, years of daily smoking, dependence (Fagerstrom Test for Nicotine Dependence, "FTND"[28]), and menthol preference.

The Perceived Health Risk Scale ("PHRS"[6,29]) assessed smokers' perceived risk for developing smoking-related health problems associated with each cigarette. Participants were instructed to assume that they would maintain their current rate of smoking while rating their health risk perceptions of each cigarette. The measure includes eight items (lung cancer, emphysema, chronic bronchitis, other cancers, heart disease, stroke, overall health risk, and risk of addiction) for which participants responded on a 1–100 visual analog scale ("very low risk" to "very high risk").

A modified version of the Cigarette Evaluation Scale ("mCES"[30,31]) was used to measure subjective cigarette effects. The "mCES" includes 15 items for which participants responded on a 7-point Likert scale to report how much they agree with each statement (not at all to extremely). Cigarette effects measured include satisfaction, taste, enjoyment of sensations in throat/chest, harshness, strength, flavor, calming, awakening, less irritable, help concentrating, hunger reduction, dizzying, nauseating, craving reduction, and enjoyment. Five reliable factors can be derived including satisfaction, psychological reward, aversion, craving reduction, and enjoyment of respiratory tract sensations.[31] An additional item was included to assess perceived level of nicotine in each cigarette. Smokers were asked, "How does the nicotine content of the study cigarette differ from your usual brand? Please answer on the following scale from 1–100, and imagine that your usual brand is 50." The visual analog scale was anchored on the ends by "much less nicotine" and "much more nicotine."

The Future Smoking Survey is a novel questionnaire created to assess how hypothetical exclusive availability of each type of cigarette would influence predicted smoking rate and interest in quitting smoking in the future. This assessment was designed to capture what participants believe they would do in a regulated marketplace (ie, if the FDA were to enact a low nicotine product standard). Participants were asked to rate on a 1–100 visual analog scale how interested they would be in quitting (not at all interested to definitely interested) at four future time points (1 month, 6 months, 1 year, and 5 years) if the cigarette they had just smoked was the only type of cigarette available to purchase. Participants were also asked to predict how many cigarettes per day they would be smoking at each of those time points.

Statistical Methods

Descriptive statistics were used to characterize the sample's demographic and smoking history variables as well as ratings of the usual brand cigarette. No statistical comparisons were made between the usual brand cigarette responses and study cigarette responses because usual brand cigarettes were always smoked first. Paired-samples *t* tests were used to compare normally distributed dependent variables across the two experimental conditions. Repeated measures ANOVA was used to test interactions between nicotine content expectancy and between-subjects variables including order of study conditions, gender, and menthol preference when appropriate. There were no significant interactions between nicotine content expectancy and order of study conditions for any of the measures. Non-normally distributed dependent variables were analyzed using the Wilcoxon matched pairs test. The McNemar's test was used to assess differences in proportions.

In addition, some participants may have misunderstood the wording of the questions on the Future Smoking Survey assessing the predicted number of cigarettes smoked per day at four future time points. Responses in the thousands suggested that participants may have mistakenly thought the question was asking the total number of cigarettes smoked over the time period assessed rather than estimating the average number of cigarettes per day they would smoke. To rectify this issue, any response over 100 cigarettes per day was excluded from analyses. Furthermore, many participants predicted smoking zero cigarettes per day in the future (at all four time points) if only these study cigarettes were available. Thus, two processes were explored with regard to the predicted number of cigarettes smoked per day data. First, the percentage of zero cigarettes per day responses was compared across study cigarette types at each future time point using a McNemar's Test. This captured the percentage of participants that predicted being abstinent. Secondly, the zero responses were removed and the remaining responses (which were adequately normally distributed) were tested using a paired-samples *t* test to explore differences in predicted cigarettes per day among participants who did not predict being abstinent.

Results

Seventy-one participants completed the study. However, three participants were excluded from analyses because they were unblinded, smoked both cigarettes during the same session or indicated smoking two "very low" nicotine cigarettes. The final sample consisted of 68 individuals (38 males, 30 females) between the ages of 19–65 years (M = 40.37, SD = 13.05). The mean number of cigarettes smoked per day was 16.53 (SD = 4.76) and years of daily smoking was 21.95 years (SD = 12.7). Sixty-six percent of the sample smoked mentholated cigarettes. The mean total score on the "FTND" was 5.85 (SD = 1.60, range = 2–9). There were no significant differences between expectancy conditions in smoking topography including total puff count, total puff volume, and interpuff interval.

The "very low" nicotine cigarette was rated as having significantly lower nicotine content (M = 23.56, SD = 23.54) than the "average" nicotine cigarette (M = 40.19, SD = 23.91), $t(67) = -5.09$, $p < .001$. However, 12 participants rated the "average" nicotine cigarette as having lower nicotine content than the "very low" nicotine cigarette, and nine participants rated the study cigarettes equally. All analyses presented below included the full sample (ie, regardless of nicotine content estimates); secondary analyses, not reported here, that focused on just those individuals (N = 47) who reported nicotine content in the expected direction ("very low" nicotine cigarette less than "average" nicotine cigarette) confirmed the reported findings.

Smokers rated the "very low" nicotine cigarette as less risky to their health overall compared to the "average" nicotine cigarette [$t(67) = -3.318$, $p = .001$]. This effect held true for all individual disease risks assessed including lung cancer [$t(67) = -4.635$, $p \leq .001$], heart disease [$t(67) = -3.953$, $p < .001$], emphysema [$t(67) = -4.521$,

$p < .001$], stroke [$t(67) = -3.738$, $p < .001$], chronic bronchitis [$t(67) = -4.001$, $p < .001$], and other cancers [$t(67) = -3.870$, $p < .001$] (Figure 1). Participants also rated the "very low" nicotine cigarette as having a lower addiction risk than the "average" nicotine cigarette [$t(67) = -2.647$, $p = .01$]. There were no significant interactions between nicotine content expectancy and gender or menthol preference for any of the perceived health risks.

Smokers also rated the "very low" nicotine cigarette as having reduced enjoyment satisfaction [$t(67) = -3.481$, $p = .001$], psychological reward [$t(67) = -2.330$, $p = .023$], and enjoyment from respiratory sensations [$t(67) = -2.913$, $p = .005$] compared to the "average" nicotine cigarette (Figure 2). The nicotine content expectancy did not have a significant effect on the aversion or craving reduction factors. There was a significant interaction between nicotine content expectancy and gender on enjoyment of respiratory tract sensations [$F(1, 66) = 4.361$, $p = .041$], such that when males were told the study cigarette contained "average" nicotine they reported greater enjoyment of respiratory tract sensations compared to when they were told the study cigarette contained "very low" nicotine and compared to females in both study conditions. A similar expectancy by gender interaction effect was marginal for psychological reward [$F(1, 66) = 3.234$, $p = .08$] and craving reduction [$F(1, 66) = 1.492$, $p = .07$]. There were no significant interactions between nicotine content expectancy and menthol preference for any of the subjective effects factors.

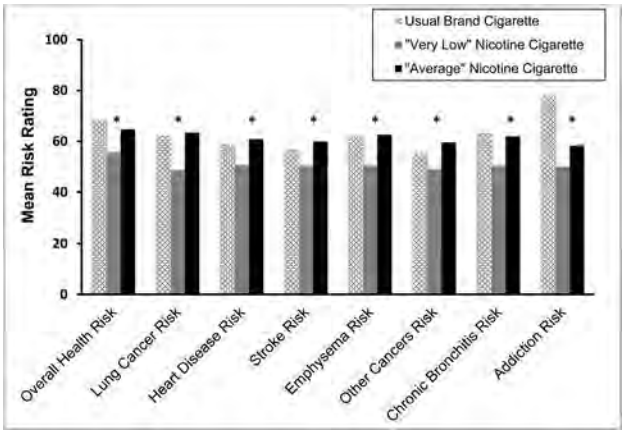

Figure 1. Ratings for all perceived health risks for usual brand cigarette and both study cigarettes. *$p \leq .01$ for "very low nicotine" versus "average" nicotine comparison. Usual brand cigarette ratings are included for reference but were not included in the analyses.

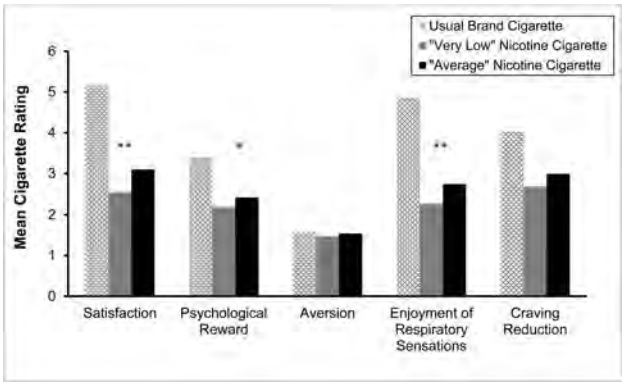

Figure 2. Results from the five factors of the cigarette evaluation scale for usual brand cigarette and both study cigarettes. *$p < .05$, **$p < .01$ for "very low nicotine" versus "average nicotine" comparison. Usual brand cigarette ratings are included for reference but were not included in the analyses.

Participants predicted greater interest in quitting smoking when considering a future in which they could only purchase the "very low" nicotine cigarette compared to the "average" nicotine cigarette at 1 month ($Z = -2.496$, $p = .013$), 6 months ($Z = -2.442$, $p = .015$), and 1 year ($Z = -2.636$, $p = .008$) (Table 1). This effect was marginal at 5 years ($Z = -1.794$, $p = .073$). Significantly more participants predicted being abstinent in 1 month and 5 years when considering exclusive availability of the "very low" nicotine cigarette compared to the "average" nicotine cigarette (Table 1). Nicotine content expectancy did not significantly impact the predicted number of cigarettes smoked per day among those who indicated they would continue to smoke.

Discussion

Independent of true nicotine content, when smokers were told a cigarette had low nicotine levels, they reported it was less harmful than a cigarette with an average amount of nicotine. Because this finding was consistent across a range of disease types and participants were instructed to assume a constant rate of smoking, it suggests this is a general misconception about how nicotine contributes to the development of smoking-related diseases. Similar to how people viewed "light" cigarettes as a healthier alternative to full flavor cigarettes,[18] smokers could misinterpret information about nicotine content and perceive VLNC cigarettes as a safer option less likely to cause harm. In light of the potential for a reduced nicotine product standard, policymakers should consider methods to mitigate this health risk misperception. Health communication campaigns reminding the public that all cigarettes, including VLNC cigarettes, are detrimental to their health should be conducted in conjunction with any policy change. Explaining that reducing nicotine could decrease the addictiveness of cigarettes but that the health risks remain similar to normal nicotine content cigarettes should be clearly conveyed to the public.

When smokers were informed that the study cigarette contained "very low" nicotine they also reported significantly fewer desirable subjective effects than when they were told it contained "average" nicotine. These findings are consistent with prior nicotine expectancy research[20,24] and are important for determining how best to frame a reduced nicotine product standard. Because smokers will receive information about potential changes in nicotine content from various sources (packaging, advertisements, word of mouth, etc.), health communication experts will need to consider how to appropriately describe VLNC cigarettes to the public. In this study, simply changing the instructions from "average" nicotine to "very low" nicotine influenced how satisfying subjects found the cigarettes and their predicted likelihood of using the product in the future. If "very low" nicotine cigarettes are perceived by smokers to be less satisfying, then this type of product description could promote greater interest in quitting. However, the perception that VLNC cigarettes are less desirable could also encourage smokers to seek alternative products. If smokers persist in using high nicotine, combusted tobacco products, for example by accessing black market cigarettes or adding nicotine to VLNC cigarettes, the potential positive impact on smoking behavior could be attenuated. A study examining the use of VLNC cigarettes for 6 weeks prior to a quit attempt found that as biomarkers of nicotine exposure increased, likely due to concurrent

Table 1. Results From the Future Smoking Survey for Usual Brand Cigarette and Both Study Cigarettes

	Usual brand cigarette	"Very low nicotine" cigarette	"Average nicotine" cigarette
Predicted quit interest	Median (range)	Median (range)	Median (range)
1 mon	14 (1–77)	53.5 (1–100)*	50 (1–100)
6 mon	20.5 (1–87)	65 (1–100)*	52 (1–100)
1 y	27.5 (1–100)	70 (1–100)**	60 (1–100)
5 y	41 (1–100)	83 (1–100)	64 (1–100)
Predicted abstinence	% Predicting zero CPD	% Predicting zero CPD	% Predicting zero CPD
1 mon	0%	14.7%*	4.4%
6 mon	0%	17.9%	9.0%
1 y	0%	21.9%	12.5%
5 y	5.9%	32.8%*	20.3%

Participants rated their predicted quit interest at four time points on a 1–100 visual analog scale. Data were not normally distributed for predicted quit interest, so median and range values are presented. Percentage of participants that predicted abstinence (smoking zero cigarettes per day) at four time points. Usual brand cigarette values are displayed for reference but were not included in analyses.
*$p < .05$; **$p < .01$.

use of non-study (ie, normal nicotine) and VLNC cigarettes, abstinence rates decreased.[32] Conversely, if current smokers who do not become abstinent switch to using non-combusted tobacco products instead of smoking, they would likely dramatically reduce their toxicant exposure.[33] Developing language to describe VLNC cigarettes that maximizes abstinence from all combusted tobacco will be an important goal for health communication experts.

Despite being perceived as less harmful, participants predicted a greater interest in quitting smoking in the future if only "very low" nicotine content cigarettes were available to purchase. Because they rated the cigarettes as less satisfactory prior to predicting their future behavior, the subjective effects rather than the perceived harmfulness of the cigarette may have had a greater impact in predicting their long term smoking behavior.

This study has several limitations. First, participants sampled the study cigarettes through smoking topography devices, which could have influenced ratings of the cigarettes. Second, the effects of a very low nicotine content expectancy on perceptions could be short-lived; future studies should measure the dependent variables repeatedly as perceptions may change with repeated exposure to the products. Third, it is possible that unmeasured characteristics of the participants' usual brand cigarettes (eg, degree of ventilation) could have moderated the impact of the expectancies. Fourth, participants were asked to predict their future behavior over the next 5 years; smokers are unlikely to be very accurate with their predictions, hence this measure only captures intentions, not actual behavior. Fifth, individual cigarettes were provided to participants with no access to cigarette packs. Therefore, this study did not address how packaging and labeling affect perceptions related to reduced nicotine cigarettes. Future research studies assessing the impact of VLNC cigarette packaging and labeling will be necessary to address this issue. Finally, future studies should utilize factorial designs to examine interactions between "actual" nicotine content and "perceived" nicotine content on smokers' perceptions of reduced nicotine cigarettes, which the present study did not capture.

In conclusion, the present study illustrates how important smokers' perceptions of nicotine content in cigarettes are on their subjective smoking ratings as well as their comprehension of the health risks associated with the product. Previous literature suggests that a very low nicotine product standard for cigarettes could have a large beneficial public health impact.[2,34] Understanding how smokers perceive very low nicotine content cigarettes is important for maximizing the public health impact of regulated reductions in the nicotine content of combusted tobacco products while minimizing unintended consequences.

Funding

This work was supported by research development funds from the University of Pittsburgh and a grant from the National Institute on Drug Abuse and FDA Center for Tobacco Products (CTP) (U54 DA031659). The funding source had no other role other than financial support. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health (NIH) or the Food and Drug Administration.

Declaration of Interests

None declared.

Acknowledgments

The authors would also like to thank Erin Goldstein, Lee Bennett, Cathy Scott, and Kristin Yahner for their assistance in data collection as well Sarah Dermody for her input on study design.

References

1. Benowitz NL, Henningfield JE. Reducing the nicotine content to make cigarettes less addictive. *Tob Control*. 2013;22(suppl 1):i14–i17.
2. Donny EC, Hatsukami DK, Benowitz NL, Sved AF, Tidey JW, Cassidy RN. Reduced nicotine product standards for combustible tobacco: building an empirical basis for effective regulation. *Prev Med*. 2014;68:17–22.
3. Hatsukami DK, Perkins KA, Lesage MG, et al. Nicotine reduction revisited: science and future directions. *Tob Control*. 2010;19(5):e1–10.
4. Donny EC, Houtsmuller E, Stitzer ML. Smoking in the absence of nicotine: behavioral, subjective and physiological effects over 11 days. *Addiction*. 2007;102(2):324–334.
5. Benowitz NL, Hall SM, Stewart S, Wilson M, Dempsey D, Jacob P III. Nicotine and carcinogen exposure with smoking of progressively reduced nicotine content cigarette. *Cancer Epidemiol Biomarkers Prev*. 2007;16(11):2479–2485.

6. Hatsukami DK, Kotlyar M, Hertsgaard LA, et al. Reduced nicotine content cigarettes: effects on toxicant exposure, dependence and cessation. *Addiction*. 2010;105(2):343–355.
7. Benowitz NL, Dains KM, Hall SM, et al. Smoking behavior and exposure to tobacco toxicants during 6 months of smoking progressively reduced nicotine content cigarettes. *Cancer Epidemiol Biomarkers Prev*. 2012;21(5):761–769.
8. Hammond D, O'Connor RJ. Reduced nicotine cigarettes: smoking behavior and biomarkers of exposure among smokers not intending to quit. *Cancer Epidemiol Biomarkers Prev*. 2014;23(10):2032–2040.
9. Donny EC, Denlinger RL, Tidey JW, et al. Randomized trial of reduced-nicotine standards for cigarettes. *N Engl J Med*. 2015;373(14):1340–1349.
10. Mercincavage M, Souprountchouk V, Tang KZ, et al. A randomized controlled trial of progressively reduced nicotine content cigarettes on smoking behaviors, biomarkers of exposure, and subjective ratings. *Cancer Epidemiol Biomarkers Prev*. 2016;25(7):1125–1133.
11. Hatsukami DK, Heishman SJ, Vogel RI, et al. Dose-response effects of spectrum research cigarettes. *Nicotine Tob Res*. 2013;15(6):1113–1121.
12. Strasser AA, Tang KZ, Tuller MD, Cappella JN. PREP advertisement features affect smokers' beliefs regarding potential harm. *Tob Control*. 2008;17(suppl 1):i32–i38.
13. Cummings KM, Hyland A, Bansal MA, Giovino GA. What do Marlboro Lights smokers know about low-tar cigarettes? *Nicotine Tob Res*. 2004;6(suppl 3):S323–S332.
14. Shadel WG, Lerman C, Cappella J, Strasser AA, Pinto A, Hornik R. Evaluating smokers' reactions to advertising for new lower nicotine quest cigarettes. *Psychol Addict Behav*. 2006;20(1):80–84.
15. Bansal-Travers M, Cummings KM, Hyland A, Brown A, Celestino P. Educating smokers about their cigarettes and nicotine medications. *Health Educ Res*. 2010;25(4):678–686.
16. Etter JF, Kozlowski LT, Perneger TV. What smokers believe about light and ultralight cigarettes. *Prev Med*. 2003;36(1):92–98.
17. Shiffman S, Pillitteri JL, Burton SL, Rohay JM, Gitchell JG. Smokers' beliefs about light and ultra light cigarettes. *Tob Control*. 2001;10(suppl 1):i17–i23.
18. Kozlowski LT, Goldberg ME, Yost BA, White EL, Sweeney CT, Pillitteri JL. Smokers' misperceptions of light and ultra-light cigarettes may keep them smoking. *Am J Prev Med*. 1998;15(1):9–16.
19. Juliano LM, Brandon TH. Effects of nicotine dose, instructional set, and outcome expectancies on the subjective effects of smoking in the presence of a stressor. *J Abnorm Psychol*. 2002;111(1):88–97.
20. Juliano LM, Fucito LM, Harrell PT. The influence of nicotine dose and nicotine dose expectancy on the cognitive and subjective effects of cigarette smoking. *Exp Clin Psychopharmacol*. 2011;19(2):105–115.
21. Kelemen WL, Kaighobadi F. Expectancy and pharmacology influence the subjective effects of nicotine in a balanced-placebo design. *Exp Clin Psychopharmacol*. 2007;15(1):93–101.
22. Perkins KA, Jacobs L, Clark L, Conklin CA, Sayette M, Wilson A. Instructions about nicotine dose influence acute responses to nasal spray. *Nicotine Tob Res*. 2004;6(6):1051–1060.
23. Mercincavage M, Smyth JM, Strasser AA, Branstetter SA. Reduced nicotine content expectancies affect initial responses to smoking. *Tob Regul Sci*. 2016;2(4):309–2136.
24. Perkins KA, Jacobs L, Ciccocioppo M, Conklin C, Sayette M, Caggiula A. The influence of instructions and nicotine dose on the subjective and reinforcing effects of smoking. *Exp Clin Psychopharmacol*. 2004;12(2):91–101.
25. Perkins KA, Grottenthaler A, Ciccocioppo MM, Conklin CA, Sayette MA, Wilson AS. Mood, nicotine, and dose expectancy effects on acute responses to nicotine spray. *Nicotine Tob Res*. 2009;11(5):540–546.
26. Darredeau C, Barrett SP. The role of nicotine content information in smokers' subjective responses to nicotine and placebo inhalers. *Hum Psychopharmacol*. 2010;25(7–8):577–581.
27. Van der Pligt J. Perceived risk and vulnerability as predictors of precautionary behaviour. *British J Health Psychol*. 1998;3(1):1–14.
28. Heatherton TF, Kozlowski LT, Frecker RC, Fagerström KO. The Fagerström Test for Nicotine Dependence: a revision of the Fagerström Tolerance Questionnaire. *Br J Addict*. 1991;86(9):1119–1127.
29. Hatsukami DK, Vogel RI, Severson HH, Jensen JA, O'Connor RJ. Perceived health risks of snus and medicinal nicotine products. *Nicotine Tob Res*. 2015;5(18):794–800.
30. Westman EC, Levin ED, Rose JE. Smoking while wearing the nicotine patch: is smoking still satisfying. *Clin Res*. 1992;40(4):A871.
31. Cappelleri JC, Bushmakin AG, Baker CL, Merikle E, Olufade AO, Gilbert DG. Confirmatory factor analyses and reliability of the modified cigarette evaluation questionnaire. *Addict Behav*. 2007;32(5):912–923.
32. Dermody SS, Donny EC, Hertsgaard LA, Hatsukami DK. Greater reductions in nicotine exposure while smoking very low nicotine content cigarettes predict smoking cessation. *Tob Control*. 2015;24(6):536–539.
33. Benowitz NL, Donny EC, Hatsukami DK. Reduced nicotine content cigarettes, e-cigarettes and the cigarette end game [published online ahead of print July 1, 2016]. *Addiction*. 2016.
34. Tengs TO, Ahmad S, Savage JM, Moore R, Gage E. The AMA proposal to mandate nicotine reduction in cigarettes: a simulation of the population health impacts. *Prev Med*. 2005;40(2):170–180.

HHS Public Access

Author manuscript

Pharmacogenet Genomics. Author manuscript; available in PMC 2018 May 01.

Published in final edited form as:

Pharmacogenet Genomics. 2017 May ; 27(5): 169–178. doi:10.1097/FPC.0000000000000271.

Variation in CYP2A6 and nicotine metabolism among two American Indian tribal groups differing in smoking patterns and risk for tobacco-related cancer

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Abstract

Objectives—The Northern Plains (NP) and Southwest (SW) American Indian populations differ in their smoking patterns and lung cancer incidence. We aimed to compare *CYP2A6* genetic variation and CYP2A6 enzyme activity (representative of the rate of nicotine metabolism) between the two tribal populations, as these have previously been associated with differences in smoking, quitting, and lung cancer risk.

Methods—American Indians (N=636) were recruited from two different tribal populations (NP in South Dakota, SW in Arizona) as part of a study conducted as part of the Collaborative to Improve Native Cancer Outcomes P50 project. A questionnaire assessed smoking-related traits and demographics. Participants were genotyped for *CYP2A6* genetic variants **1B*, **2, *4*, **7*, **9*, **12*, **17,* and **35*. Plasma and/or saliva samples were used to measure nicotine's metabolites cotinine and 3′-hydroxycotinine and determine CYP2A6 activity (3′-hydroxcotinine/cotinine, i.e. the nicotine metabolite ratio, NMR).

Results—The overall frequency of genetically reduced nicotine metabolizers, those with *CYP2A6* decrease- or loss-of-function alleles, was lower in the NP compared to the SW (P=0.0006). *CYP2A6* genotype was associated with NMR in both tribal groups (NP P<0.001, SW P=0.04). Notably, the rate of nicotine metabolism was higher in NP compared to SW smokers

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Conflicts of Interest: Dr. Tyndale has consulted for Apotex. The remaining authors declare no conflicts of interest.

(*P*=0.03), and in comparison to other ethnic groups in the United States. Of the variables studied, *CYP2A6* genotype was the only variable to significantly independently influence NMR among smokers in both tribal populations (NP *P*<0.001, SW *P*=0.05).

Conclusions—Unique *CYP2A6* allelic patterns and rates of nicotine metabolism among these American Indian populations suggest different risks for smoking and tobacco-related disease.

Keywords

CYP2A6; genetic variation; nicotine metabolism; nicotine metabolite ratio (NMR); smoking

INTRODUCTION

Patterns of commercial tobacco use in the United States (U.S.) differ among ethnic groups, with cigarette smoking being more prevalent in American Indian and Alaska Native (AI/AN) populations compared to other U.S. populations [1]. However, between different AI/AN tribal populations, prevalence and smoking patterns are also variable, for example between the Northern Plains (NP) and Southwestern (SW) tribal populations [2, 3]. Despite most AI/ANs wanting to quit smoking, and making quit attempts [4–6], the prevalence of smoking is 50% in the NP tribal population of South Dakota while only being 14% in the SW tribal population of Arizona [2], and cigarette consumption is higher in the NP relative to the SW (13 vs. 7 cigarettes per day, CPD) [3]. Consistent with this, rates of lung cancer incidence and mortality, to which cigarette smoking has been causally linked, are more than 6 times higher in the NP compared to the SW tribal population [7, 8]. The underlying reasons for these large differences in tribal smoking and disease risk, whether biological and/or social, are unknown.

One biological influence on smoking is the activity of the hepatic CYP2A6 (cytochrome P450 2A6) enzyme, which varies substantially between individuals and ethnicities [9]. Nicotine, the primary psychoactive component present in cigarette smoke, is metabolically inactivated by CYP2A6 [10] with the major pathway of inactivation being conversion of nicotine to cotinine (COT) [11]. COT is further metabolized to *trans*-3′-hydroxycotinine (3HC) exclusively by CYP2A6 [12]. Interindividual variation in CYP2A6 enzymatic activity, and thus the rate of nicotine metabolism and clearance, largely results from genetic variation in *CYP2A6*, the highly polymorphic gene encoding the CYP2A6 enzyme (variants characterized to date found at http://www.cypalleles.ki.se/cyp2a6.htm). Regular daily smokers alter their levels and intensity of smoking to titrate their nicotine intake such that they maintain desired nicotine levels in the body [13]. Accordingly, *CYP2A6* genetic variation and altered rates of nicotine metabolism are associated with differences in smoking behaviors [9]. Smokers with slower rates of nicotine metabolism, those possessing decrease and/or loss-of-function *CYP2A6* alleles (referred to as reduced metabolizers), often have lower tobacco consumption, dependence, and difficulty quitting smoking compared to faster metabolizers [14–16]. Among heavy smokers (≥10 CPD), genetically reduced metabolizers smoke fewer CPD compared to smokers with wild-type *CYP2A6* genotypes [14]. However, in lighter smoking populations (<10 CPD), measurement of CPD is not a sensitive measure of tobacco consumption [17, 18]; while light smokers may consume the same number of CPD, they can differ substantially in their smoking topography (puff volume, duration,

velocity), and thus overall tobacco consumption [19]. Measuring urinary total nicotine equivalents (TNE) is a considerably more precise biomarker of nicotine dose compared to CPD and is the current gold standard for assessing intake [20, 21]. TNE was lower among Alaska Native light smokers with reduce-of-function *CYP2A6* genotypes, and lower rates of nicotine metabolism, despite no differences in CPD [17].

In addition to *CYP2A6* genotype, enzyme activity itself is associated with smoking behaviors [22, 23]. CYP2A6 enzymatic activity can be determined in smokers using a ratio of nicotine's metabolites 3HC/COT (nicotine metabolite ratio, NMR), which is highly correlated to the rate of nicotine metabolism and clearance [24]. The NMR is a validated biomarker of CYP2A6 activity due to its stability over time and measurement consistency among heavy and light smokers [25, 26]. Previous studies have confirmed the strong concordance between *CYP2A6* genotype and NMR in multiple populations and ethnic groups suggesting similar impacts on smoking and lung cancer [18, 27].

Lung cancer risk is also associated with *CYP2A6* genetic variation. Smoking fewer CPD, which has been observed among genetically reduced compared to normal nicotine metabolizers [14], is associated with lower lung cancer risk [28], however the relationship between CYP2A6 and lung cancer risk remains even when controlling for cigarette consumption [29]. One reason is that CYP2A6 can also metabolically activate procarcinogenic tobacco-specific nitrosamines; thus being a slower CYP2A6 metabolizer can reduce both tobacco consumption and procarcinogen activation resulting in lower lung cancer risk [30].

Although the impact of individual *CYP2A6* alleles/genotypes on the rate of nicotine metabolism has been consistent across ethnicities [18, 27, 31], patterns of *CYP2A6* genetic variation and nicotine metabolism vary substantially between different ethnic groups. [32, 33]. [27, 34, 35]. To date, no studies have investigated *CYP2A6* genetic variation, and characterized associations between *CYP2A6* genotype and the rate of enzymatic activity, among American Indian populations, let alone compared two independent tribal groups with contrasting patterns of smoking. Given the distinct patterns of *CYP2A6* genetic variation among different ethnic groups, it is plausible that the NP and SW tribal populations exhibit unique patterns of variation at this locus. Therefore, the aim of the current study was to compare *CYP2A6* genetic variation and the rate of nicotine metabolism between the NP and SW tribal populations.

MATERIALS AND METHODS

Study design

Members of American Indian tribal groups were recruited between 2012 and 2014 in South Dakota and Arizona for a study entitled "Topography and Genetics of Smoking and Nicotine Dependence in American Indians", one of five major research studies conducted by the Collaborative to Improve Native Cancer Outcomes. Recruitment in the NP occurred from a random subset of participants in an earlier study of community health [36]. Recruitment in the SW was conducted using respondent driven sampling among American Indian friends and family members of tribal participants in an earlier randomized clinical trial in the greater

Phoenix metropolitan area [37]. Both NP and SW subsamples were stratified by sex and smoking status. The final cohort comprised N=636 American Indians aged 20–88 years, with N=426 in the NP and N=210 in the SW groups. The urban SW and the predominantly rural NP tribal populations are culturally and linguistically distinct, and have considerably different historical experiences [38].

Data were collected by trained research staff who were tribal members. Biological samples (blood or saliva) were collected from all participants to be used for genotyping and phenotyping. Personal interviews were conducted, and questionnaires were administered to all participants. Ethical approval for all study procedures was obtained from the institutional review boards of the Great Plains Area Indian Health Service, the University of Toronto, the University of Washington, and MedStar Health Research Institute, and passed through individual tribal approval processes. All participants provided informed consent.

Measures

Data were obtained on age, gender, body mass index (BMI), smoking status, duration of smoking, cigarettes smoked per day (CPD), nicotine dependence scores (Fagerström Test for Nicotine Dependence, FTND; Hooked On Nicotine Checklist, HONC), and ceremonial traditional tobacco use. Levels of COT and 3HC were measured in blood or saliva samples using liquid chromatography tandem mass spectrometry, as previously described [39]. Our phenotypic measure of CYP2A6 activity, NMR, was defined as the ratio of nicotine's metabolites, 3HC/COT [24].

Relatedness was determined for all participants based on self-report and genetic analyses. When nuclear families were enrolled, only data on parents (founders) were used to determine allele frequencies; when sibships were enrolled, only one randomly chosen sibling was used. In addition to self-report, relatedness was also assessed by identity-by-descent (IBD) values. These data showed an excellent concordance with the self-reported family data. For individuals who had enrolled as independent, we assessed potential cryptic relatedness, and excluded one member of each pair with IBD values of 0.25 or greater. IBD values were computed using SNP & Variation Suite (SVS; Golden Helix, Inc.). Only unrelated participants were included in the comparison of *CYP2A6* gene variant frequencies between the two tribal populations. Only self-reported smokers with COT levels above 10 ng/ml have been included in the NMR analyses to ensure smokers had sufficient COT levels that 3HC was quantifiable and formation dependent [39, 40]. NMR is used as a biomarker of CYP2A6 activity in current regular smokers only as steady state levels of cotinine and 3HC, achieved through regular cigarette smoking, are necessary for accurate measurement of CYP2A6 activity; this is not a feasible measurement of CYP2A6 activity in non-smokers in this study [41].

CYP2A6 Genotyping Assays

DNA was extracted from saliva or blood, and DNA from N=634 participants (NP N=426, SW n=208) was successfully genotyped for the following *CYP2A6* alleles: **1B*, **2*, **4*, **7*, **9*, **12*, **17*, and **35*. Genotyping was performed using a two-step allele-specific polymerase chain reaction approach, or an allele-specific TaqMan single nucleotide

polymorphism genotyping assay (Applied Biosystems) and real-time polymerase chain reaction, as described previously [42]. The *CYP2A6* alleles **2*, **4*, **7*, **9*, **12*, **17*, and **35* have been associated with a decrease or loss of CYP2A6 enzyme activity and rates of nicotine metabolism [18, 43, 44], whereas the **1B* allele associates with faster nicotine metabolism [45]. We have grouped participants as "normal" or "reduced" CYP2A6 metabolizers based on the predicted impact of their *CYP2A6* genotype. Normal metabolizers possessed no *CYP2A6* decrease- or loss-of-function genetic variants, whereas reduced metabolizers possessed one or more copies of these alleles.

Statistical analyses

NMR was non-normally distributed, thus nonparametric statistical tests were used. Chi-squared tests were used to determine Hardy-Weinberg equilibrium, and to compare the frequencies of *CYP2A6* genetic variants, and the overall proportion of subjects who were genetically reduced metabolizers, between the NP and SW tribal populations. We used Kruskal-Wallis and Dunn's Multiple Comparisons tests to analyze the association of *CYP2A6* genotype with NMR. Mann-Whitney tests were conducted to compare NMR between genetically normal versus reduced metabolizers within each tribal population and to compare overall NMR between the NP and SW populations.

Linear regression models were run among smokers with COT>10 to determine the percentage of variation in NMR (the output variable) accounted for. Our first model included the variables *CYP2A6* genotype, gender, age, BMI, CPD, ceremonial traditional tobacco use, and tribal population (i.e. NP or SW). We also ran separate regression models for each tribal population, in which model variables included *CYP2A6* genotype and BMI. In each model, *CYP2A6* genotype was coded as 0 for individuals with no known CYP2A6 reduced activity alleles (i.e. **1A/*1A*, **1A/*1B*, **1B/*1B* genotypes), −1 for those possessing one decrease-of-function allele (i.e. **1/*9* and **1/*12* genotypes), and −2 for those possessing one or more loss-of-function or two decrease-of-function alleles (i.e. **1/*2*, **1/*4*, and **9/*9* genotypes), as done previously [46]. A linear regression model of NMR was also run in wild-type (*CYP2A6*1/*1*, i.e. individuals who do not possess any known *CYP2A6* reduce activity genetic variants) smokers only to assess the impact of the increase-of-function *CYP2A6*1B* genotype and tribal population. Additionally, a linear regression model was used to test if *CYP2A6*1B* genotype interacts with tribal population to influence NMR. The single predictors (*CYP2A6*1B* genotype and tribal population) were entered into block 1, and the interaction term (*CYP2A6*1B* genotype x tribal population) was entered into block 2. In both models, *CYP2A6* genotype was coded as 0 for individuals with **1A/*1A* genotype, 1 for those possessing **1A/*1B* genotype, and 2 for those possessing **1B/*1B* genotype [45]. Analyses were conducted with GraphPad Prism (v6.0) and SPSS (v22), and statistical tests were considered significant for $P<0.05$.

RESULTS

Frequencies of *CYP2A6* alleles and genetically reduced nicotine metabolizers

CYP2A6 genotype frequencies within each tribal population did not significantly deviate from Hardy-Weinberg equilibrium ($P>0.05$). The two NP and SW tribal populations

exhibited distinct frequencies of *CYP2A6* variant alleles from one another and compared to other ethnic groups (Table 1). The frequency of the gain-of-function **1B* allele was significantly higher in the NP than the SW (NP 69.7% vs. SW 61.6%, P=0.01), whereas, prevalence of the decrease-of-function **9* allele was significantly lower in the NP compared to the SW (NP 11.9% vs. SW 20.9%, P=0.0002). Several alleles that have been found in other ethnic groups were not present in the NP and SW tribal populations (NP **7*, **17*, **35*; SW **7*, **17*). Next we categorized individuals into "normal" or "reduced" CYP2A6 activity groups based on genotype, with reduced metabolizers defined as individuals who possess any decrease- or loss-of-function alleles; this included the following genotypes: **1/*2*, **1/*4*, **1/*9*, **9/*9*, **1/*12*, and **1/*35*. A smaller proportion of the NP tribal population were *CYP2A6* genotype reduced nicotine metabolizers compared to the SW tribal population (NP 27.5% vs. SW 41.8%, P=0.0006).

Associations of *CYP2A6* genotype with CYP2A6 activity (NMR)

We assessed the rate of CYP2A6 activity, determined by the NMR, among smokers to determine if the functional impact of the change in each variant *CYP2A6* allele was consistent with the impact observed in other populations, and in *in vitro* expression systems [47]. The *CYP2A6*1B* allele had previously been associated with an increased rate of overall nicotine clearance and NMR *in vivo* [45], and we observed higher NMR among smokers in both the NP (P=0.02) and SW (P=0.002) tribal populations who possessed the **1B* allele relative to the wild-type **1A* allele (Fig. 1; three-group comparison across **1A/*1A*, **1A/*1B*, and **1B/*1B* genotypes). As many decrease- and loss-of-function alleles exist on a variety of **1A* or **1B* backbones, we have grouped these as the **1/*1* wild-type group for comparisons to the *CYP2A6* genotype reduce activity group (a compilation of all *CYP2A6* decrease- and loss-of-function alleles).

CYP2A6 genotype was associated with CYP2A6 activity such that smokers possessing one or more decrease- or loss-of-function alleles exhibited lower mean NMR compared to the **1/*1* reference group (NP P<0.0001, SW P=0.04; Fig. 2). We also observed the presence of a gene-dose effect with the prevalent decrease-of-function **9* allele in both the NP and SW tribal populations. In the NP tribal population, relative to the wild-type group (**1/*1* genotype), those with one copy of the **9* allele (**1/*9* genotype) had 63.4% CYP2A6 activity, and those with two copies of **9* (**9/*9* genotype) had 29.6% CYP2A6 activity (Fig. 2). Similarly, in the SW tribal population, smokers with one and two copies of **9* exhibited 84.0% and 52.7% CYP2A6 activity, respectively (Fig. 2).

Comparison of the rate of nicotine metabolism across populations

We compared the rate of nicotine metabolism (i.e. CYP2A6 activity), determined by NMR, between NP and SW smokers, and with additional populations of different ethnic backgrounds. Among all smokers (wild-type and *CYP2A6* genotype reduced metabolizers combined), NP smokers had a faster mean rate of nicotine metabolism relative to SW smokers (P=0.03; Figs. 3a, Supplementary Figure 1a). NP smokers also had a higher mean rate of nicotine metabolism compared to smokers from other ethnic groups, including Alaska Native, Caucasian, and African American smokers, whose NMR had been previously assessed [18, 27, 48]. When analyses were restricted to wild-type smokers only (those who

do not possess any known *CYP2A6* decrease- or loss-of-function alleles) to avoid the confound of higher or lower frequencies of CYP2A6 genotype reduced nicotine metabolizers between populations, the rate of nicotine metabolism remained higher among NP smokers compared to SW smokers (P=0.003), and compared to smokers from other ethnic groups (Figs. 3b, Supplementary Figure 1b). In addition, the rate of nicotine metabolism was again higher among NP relative to SW smokers when analyses were restricted to the **1B/*1B* group (P=0.02; Fig 1).

Variables that influence the rate of nicotine metabolism

Using linear regression modeling, we investigated the proportion of variation in the rate of nicotine metabolism (NMR) that was attributable to different predictor variables among smokers in each tribal population, while controlling for the influence of other variables. Both tribal populations were included in a model of NMR in which *CYP2A6* genotype, gender, age, ceremonial traditional tobacco use, BMI, CPD, and tribal population (i.e. NP and SW) were assessed as predictor variables of NMR. Only *CYP2A6* genotype (P<0.001), BMI (P=0.03), and tribal population (P=0.06) each independently accounted for >1% of the variation in NMR (Table 2a). In a second model we assessed predictors of NMR separately in the NP and SW tribal populations, including only the variables *CYP2A6* genotype and BMI (Table 2b and c). These models indicated that, of the variables studied in both tribal populations, *CYP2A6* genotype was the only significant independent predictor of NMR among smokers, accounting for 19.71% (P<0.001) and 8.12% (P=0.05) of the variation in NMR in the NP and SW tribal populations, respectively (Table 2).

As we have observed that NP smokers have a higher overall NMR compared to SW smokers, we aimed to determine if this observation was independent of the effect of the increase-of-function *CYP2A6*1B* allele, which was expressed at a higher frequency in the NP compared to the SW tribal population. We ran a linear regression model for NMR among wild-type smokers only in which *CYP2A6*1B* genotype and tribal population were included as predictor variables. Both variables independently accounted for ≥6% of the variation in NMR (*CYP2A6*1B* genotype, 7.34%, P=0.0006; tribal population, 6.00%, P=0.01; Table 3a). Additionally, using linear regression analyses, we assessed if there was a significant interaction between *CYP2A6*1B* genotype and tribal population in influencing NMR. The interaction term (genotype x tribal population) was not significant (P=0.72, model R^2 change=0.001; Table 3b), suggesting that the relationship between *CYP2A6*1B* genotype and NMR is similar in both tribal populations.

Associations between smoking behaviors and *CYP2A6* genotype and activity

Relationships between *CYP2A6* genotype and NMR and smoking behaviors were assessed. There was no difference in the proportion of current and former smokers who were *CYP2A6* genotype reduced metabolizers in either the NP or SW tribal populations (NP current smokers 31.7%, former smokers 26.7%, P=0.37; SW current smokers 44.9%, former smokers 41.5%, P=0.71). There was no association between the following smoking behaviors and *CYP2A6* genotype or NMR among either NP or SW self-reported smokers: mean number of CPD, FTND score, HONC score, and duration of smoking (Supplementary

Table 1a). Parallel analyses were run in which smokers were defined by the more stringent cut point of COT>10; these analyses yielded similar results (Supplementary Table 1b).

DISCUSSION

We have demonstrated that the NP and SW tribal populations are genetically distinct at the *CYP2A6* locus, with the NP population being comprised of a lower frequency of *CYP2A6* genotype reduced metabolizers relative to the SW tribal population, while also exhibiting distinct individual allele frequencies. In both cohorts, *CYP2A6* genotype was associated with our phenotypic measure of CYP2A6 activity and rate of nicotine metabolism, NMR. Notably, NP smokers exhibited a faster overall rate of nicotine metabolism compared to smokers in the SW tribal population and other ethnic groups, even among the *CYP2A6* wild-type subgroup. *CYP2A6* genotype and NMR were not significantly associated with smoking behaviors in either tribal population.

The variety of *CYP2A6* alleles that were investigated in this study (**1B*, **2*, **4*, **7*, **9*, **12*, **17*, **35*) were representative of those with a functional impact and those which were prevalent among different ethnic groups [18, 27, 44]. For example, as illustrated in Table 1, common *CYP2A6* alleles (frequency >1%) among different ethnic populations include: Caucasians **1B*, **2*, **4*, **9, *12*; African Americans **1B, *2*, **4*, **9, *17, *35*; Asians **1B, *4, *7, *9*; Alaska Natives **1B, *4, *9* [49]. Choosing to investigate this variety of alleles allowed us to compare *CYP2A6* genetic variation between the two populations, and characterize the tribal populations with respect to other ethnic groups. We had expected that the pattern of *CYP2A6* allele frequencies among both the NP and SW tribal populations would most resemble that of Asian populations, as it is postulated that the NP and SW tribal populations have common Asian ancestral origins [50]. However, the *CYP2A6*7* allele, which is observed almost exclusively in those of Asian descent, was not present in either tribal population, and both the NP and SW have much lower frequencies of the *CYP2A6*4* allele that is common in Asians (see Table 1). Additionally, we observed significantly different *CYP2A6* allele frequencies between the NP and SW tribal populations, suggesting that, if these two populations share common ancestry, there have been incidences of genetic divergence between these two populations. There are several potential explanations for their unique genetic patterns. As each tribal population may have been established by a relatively small sample of original ancestors, a founder effect could have contributed to the genetic divergence between tribal populations, as well as from their ancestors [51]. Additionally, because the tribal populations have been geographically separated, forces such as lack of gene flow, random genetic drift, and selective pressures could contribute to development of these unique allele frequency patterns [52, 53].

Not only do the NP and SW tribal populations exhibit different patterns of *CYP2A6* allele frequencies, but more specifically, the NP population comprises fewer *CYP2A6* genotype reduced nicotine metabolizers. Considering the strong association that we observed between *CYP2A6* genotype and activity, this suggests that NP smokers will have a faster rate of nicotine metabolism. As previously stated, faster metabolism has been associated with heavier smoking and higher lung cancer risk [14–16, 29], consistent with characteristics of the NP compared to the SW tribal population [3, 7, 8]. Phenotypic measurements of

CYP2A6 activity also confirmed that the NP tribal population has an elevated rate of nicotine metabolism relative to the SW tribal population, and compared to Alaska Native, Caucasian, and African American populations. This observation was present in the overall population of smokers, but also when excluding smokers possessing known *CYP2A6* decrease- or loss-of-function alleles. The latter finding suggests that the observed elevated rate of nicotine metabolism among NP smokers was *not* a result of a lower frequency of reduced metabolizers, but rather other factors are contributing to this, as discussed below. Faster nicotine metabolism may contribute to an elevated risk for smoking and tobacco-related disease in the NP relative to the SW, as well as in comparison to other ethnic populations in the United States.

In order to investigate potential contributors to NMR, we conducted linear regression modeling in the tribal populations. *CYP2A6* genotype and BMI were the only significant independent contributors to NMR in the combined population sample. However, in this model, tribal population itself trended toward significance, which suggests that there may be unaccounted for differences between the regional tribal populations that explain the observed higher NMR among the NP compared to the SW tribal population. When we investigated each tribal population separately, we found that *CYP2A6* genotype was the only significant independent contributor to NMR in both the NP and SW tribal populations. Therefore, we investigated if a possible explanation for the high NMR in the NP tribal population could be their relatively high frequency of the *CYP2A6*1B* allele (69.7%) compared to the **1B* allele frequency in the SW tribal population (61.6%) and other ethnic groups (Alaska Natives 65%, Caucasians 32%, African Americans 14%, approximately) [27, 54]. *CYP2A6*1B* was associated with higher NMR in both tribal populations, and in previous studies, with the mechanism believed to be through improving *CYP2A6* mRNA stabilization, leading to increased CYP2A6 enzyme expression and activity [45, 55]. However, as NP smokers possessing the **1B/*1B* genotype had significantly higher NMR compared to SW smokers with the same genotype, this suggests that the tribal differences in NMR are not resulting from the higher frequency of **1B* in the NP compared to the SW tribal population. As further evidence of this, our linear regression model run in wild-type smokers only indicated that *CYP2A6*1B* genotype and tribal population were significant independent predictors of NMR. This suggests that, independent of *CYP2A6*1B* genotype, the NP tribal population exhibits higher NMR than the SW tribal population. We also confirmed that there was no interaction between *CYP2A6*1B* genotype and tribal population in influencing NMR, suggesting that the relationship between *CYP2A6*1B* genotype and NMR is similar in both tribal populations and therefore other factors, unrelated to *CYP2A6*1B,* are contributing to tribal differences in NMR.

Considering the highly polymorphic nature of the *CYP2A6* gene (http://www.cypalleles.ki.se/cyp2a6.htm), there may be novel *CYP2A6* genetic variants present in the NP tribal population that act to increase *CYP2A6* gene and protein expression, and/or enzyme activity. For example, in the NP population there may be undetected genetic variation present at the 5′ promoter region of the *CYP2A6* gene that interrupts the binding site for an inhibitory transcription factor, such as C/EBPβ, or creates a binding site for an activating transcription factor, such as HNF4α thus decreasing or increasing CYP2A6 levels [56]. This may result in greater *CYP2A6* promoter activity and thus gene transcription.

Variation in regions 3′ of the *CYP2A6* gene, similar to the **1B* allele, may contribute to increased mRNA stability and thus increased protein expression [55], contributing to higher NMR among NP relative to SW smokers. The significantly higher frequency of *CYP2A6*9* in the SW compared to NP supports the concept of ethnicity, or tribal group, specific genetic variation and variant distribution. Assessment of both common and rare variation at the *CYP2A6* locus, and in upstream and downstream sequence, by whole *CYP2A6* gene sequencing would improve our understanding of the inter-tribe differences in CYP2A6 and nicotine metabolism. Additionally, environmental factors can also modulate CYP2A6 activity and the rate of nicotine metabolism. For example, CYP2A6 is inducible by dietary and pharmacological agents such as broccoli, rifampin, and phenobarbital [57–59], and therefore potentially by other dietary components of the NP tribal population.

Implications of having a faster rate of nicotine metabolism, as seen in the NP tribal population, include having greater tobacco consumption, nicotine dependence scores, difficulty quitting smoking, and lung cancer risk [14–16, 29]. Greater activation of tobacco-specific procarcinogens, such as nitrosamines, due to faster CYP2A6 activity, may explain the higher lung cancer incidence and mortality rates in the NP compared to the SW [7, 8, 30], however our study did not specifically assess this.

When investigating smoking behaviors, we observed that NP smokers consume more CPD (7 vs. 4) [unpublished], have higher nicotine dependence scores (FTND 2.0 vs. 1.8, HONC 4.5 vs. 3.7) [unpublished], and have longer durations of smoking (23 vs. 20 years) [unpublished] compared to SW smokers. However, we did not observe an association between *CYP2A6* genotype or NMR with smoking behaviors in either tribal population. This highlights some limitations of our study. First, this lack of association may have resulted from limited statistical power to detect smoking behavior differences between *CYP2A6* genotype or NMR groups due to low sample sizes, particularly of the smokers. The sample size calculations were based on a separate candidate-gene study, conducted as part of the broader “Topography and Genetics of Smoking and Nicotine Dependence in American Indians” study. Additionally, we were limited by available biomarkers and measures of tobacco consumption. The use of a more sensitive biomarker of tobacco consumption, such as TNE, may in the future allow us to detect subtle differences in smoking among these two light smoking populations. Moreover, titration of nicotine intake by CPD according to rate of nicotine metabolism may not be observed in these AI/AN populations, similarly to African American smokers [18, 60], but titration may be detected with TNE as measure of consumption, as seen among Alaska Native smokers [17].

In conclusion, we have identified distinct patterns of *CYP2A6* genetic variation and rates of nicotine metabolism between two AI/AN tribal populations with different smoking patterns and risk for lung cancer. Given the association between faster nicotine metabolism and greater smoking and lung cancer risk, these findings suggest that CYP2A6 may contribute to an elevated risk for smoking and tobacco-related disease in the NP compared to the SW, and compared to other ethnic populations in the United States. Future work should focus on determining the cause(s) of the faster nicotine metabolism in the NP and assess any causal relationships between their increased rate of metabolism and lung cancer risk and

differences in smoking behaviors including cessation, which may in turn inform strategies to mitigate the elevated risk [16, 22].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding: We acknowledge the support of the Endowed Chair in Addictions for the Department of Psychiatry (R.F. Tyndale), National Institutes of Health (NIH) Pharmacogenomics Research Network grant DA020830, Canadian Institutes of Health Research grant TMH109787, the Centre for Addiction and Mental Health (CAMH), the Campbell Family Mental Health Research Institute of CAMH, the CAMH Foundation, the Canada Foundation for Innovation (#20289 and #16014), and the Ontario Ministry of Research and Innovation. This research was performed under the auspices of both the Collaborative to Improve Native Cancer Outcomes, a P50 program project sponsored by the National Cancer Institute of the NIH (#P50CA148110; PIs Buchwald and Henderson), and the Native American Research Centers for Health Initiative of the Indian Health Service and NIH (#S06GM092240).

This research was performed under the auspices of the Collaborative to Improve Native Cancer Outcomes (CINCO), a P50 program project sponsored by the National Cancer Institute (#P50CA148110; PIs Buchwald and Henderson). CINCO includes Buchwald D, Flum DR, Garroutte EM, Gonzales AA, Henderson JA, Nez Henderson P, Patrick DL, Tu SP, Winer RL. Additionally, this research was further supported by a grant from the Native American Research Centers for Health initiative of the Indian Health Service and NIH (#S06GM092240; PI Henderson). We acknowledge Maria Novalen and Bin Zhao for performing the analytical chemistry.

References

1. Centers for Disease C, Prevention. Prevalence of cigarette use among 14 racial/ethnic populations--United States, 1999–2001. MMWR Morbidity and mortality weekly report. 2004; 53(3):49–52. [PubMed: 14749612]
2. Nez Henderson P, Jacobsen C, Beals J, Team A-S. Correlates of cigarette smoking among selected Southwest and Northern plains tribal groups: the AI-SUPERPFP Study. American journal of public health. 2005; 95(5):867–72. [PubMed: 15855467]
3. Eichner JE, Wang W, Zhang Y, Lee ET, Welty TK. Tobacco use and cardiovascular disease among American Indians: the strong heart study. International journal of environmental research and public health. 2010; 7(10):3816–30. [PubMed: 21139862]
4. Jamal A, Agaku IT, O'Connor E, King BA, Kenemer JB, Neff L. Current cigarette smoking among adults--United States, 2005–2013. MMWR Morbidity and mortality weekly report. 2014; 63(47): 1108–12. [PubMed: 25426653]
5. Gohdes D, Harwell TS, Cummings S, Moore KR, Smilie JG, Helgerson SD. Smoking cessation and prevention: an urgent public health priority for American Indians in the Northern Plains. Public health reports. 2002; 117(3):281–90. [PubMed: 12432139]
6. Forster JL, Rhodes KL, Poupart J, Baker LO, Davey C. American Indian Community Tobacco Project Steering C. Patterns of tobacco use in a sample of American Indians in Minneapolis-St. Paul. Nicotine & tobacco research : official journal of the Society for Research on Nicotine and Tobacco. 2007; 9(Suppl 1):S29–37. [PubMed: 17365724]
7. Bliss A, Cobb N, Solomon T, Cravatt K, Jim MA, Marshall L, et al. Lung cancer incidence among American Indians and Alaska Natives in the United States, 1999–2004. Cancer. 2008; 113(5 Suppl): 1168–78. [PubMed: 18720387]
8. Plescia M, Henley SJ, Pate A, Underwood JM, Rhodes K. Lung cancer deaths among American Indians and Alaska Natives, 1990–2009. American journal of public health. 2014; 104(Suppl 3):S388–95. [PubMed: 24754613]
9. Malaiyandi V, Sellers EM, Tyndale RF. Implications of CYP2A6 genetic variation for smoking behaviors and nicotine dependence. Clinical pharmacology and therapeutics. 2005; 77(3):145–58. [PubMed: 15735609]

10. Benowitz NL, Jacob P 3rd. Metabolism of nicotine to cotinine studied by a dual stable isotope method. Clinical pharmacology and therapeutics. 1994; 56(5):483–93. [PubMed: 7955812]
11. Messina ES, Tyndale RF, Sellers EM. A major role for CYP2A6 in nicotine C-oxidation by human liver microsomes. The Journal of pharmacology and experimental therapeutics. 1997; 282(3): 1608–14. [PubMed: 9316878]
12. Nakajima M, Yamamoto T, Nunoya K, Yokoi T, Nagashima K, Inoue K, et al. Characterization of CYP2A6 involved in 3′-hydroxylation of cotinine in human liver microsomes. The Journal of pharmacology and experimental therapeutics. 1996; 277(2):1010–5. [PubMed: 8627511]
13. McMorrow MJ, Foxx RM. Nicotine's role in smoking: an analysis of nicotine regulation. Psychol Bull. 1983; 93(2):302–27. [PubMed: 6844475]
14. Schoedel KA, Hoffmann EB, Rao Y, Sellers EM, Tyndale RF. Ethnic variation in CYP2A6 and association of genetically slow nicotine metabolism and smoking in adult Caucasians. Pharmacogenetics. 2004; 14(9):615–26. [PubMed: 15475735]
15. Schnoll RA, Patterson F, Wileyto EP, Tyndale RF, Benowitz N, Lerman C. Nicotine metabolic rate predicts successful smoking cessation with transdermal nicotine: a validation study. Pharmacology, biochemistry, and behavior. 2009; 92(1):6–11.
16. Wassenaar CA, Dong Q, Wei Q, Amos CI, Spitz MR, Tyndale RF. Relationship between CYP2A6 and CHRNA5-CHRNA3-CHRNB4 variation and smoking behaviors and lung cancer risk. Journal of the National Cancer Institute. 2011; 103(17):1342–6. [PubMed: 21747048]
17. Zhu AZ, Binnington MJ, Renner CC, Lanier AP, Hatsukami DK, Stepanov I, et al. Alaska Native smokers and smokeless tobacco users with slower CYP2A6 activity have lower tobacco consumption, lower tobacco-specific nitrosamine exposure and lower tobacco-specific nitrosamine bioactivation. Carcinogenesis. 2013; 34(1):93–101. [PubMed: 23027621]
18. Ho MK, Mwenifumbo JC, Al Koudsi N, Okuyemi KS, Ahluwalia JS, Benowitz NL, et al. Association of nicotine metabolite ratio and CYP2A6 genotype with smoking cessation treatment in African-American light smokers. Clinical pharmacology and therapeutics. 2009; 85(6):635–43. [PubMed: 19279561]
19. Bridges RB, Combs JG, Humble JW, Turbek JA, Rehm SR, Haley NJ. Puffing topography as a determinant of smoke exposure. Pharmacology, biochemistry, and behavior. 1990; 37(1):29–39.
20. Benowitz NL, Jacob P 3rd, Fong I, Gupta S. Nicotine metabolic profile in man: comparison of cigarette smoking and transdermal nicotine. The Journal of pharmacology and experimental therapeutics. 1994; 268(1):296–303. [PubMed: 8301571]
21. Scherer G, Engl J, Urban M, Gilch G, Janket D, Riedel K. Relationship between machine-derived smoke yields and biomarkers in cigarette smokers in Germany. Regul Toxicol Pharmacol. 2007; 47(2):171–83. [PubMed: 17034917]
22. Lerman C, Schnoll RA, Hawk LW Jr, Cinciripini P, George TP, Wileyto EP, et al. Use of the nicotine metabolite ratio as a genetically informed biomarker of response to nicotine patch or varenicline for smoking cessation: a randomised, double-blind placebo-controlled trial. The Lancet Respiratory medicine. 2015; 3(2):131–8. [PubMed: 25588294]
23. Falcone M, Jepson C, Benowitz N, Bergen AW, Pinto A, Wileyto EP, et al. Association of the nicotine metabolite ratio and CHRNA5/CHRNA3 polymorphisms with smoking rate among treatment-seeking smokers. Nicotine & tobacco research : official journal of the Society for Research on Nicotine and Tobacco. 2011; 13(6):498–503. [PubMed: 21385908]
24. Dempsey D, Tutka P, Jacob P 3rd, Allen F, Schoedel K, Tyndale RF, et al. Nicotine metabolite ratio as an index of cytochrome P450 2A6 metabolic activity. Clinical pharmacology and therapeutics. 2004; 76(1):64–72. [PubMed: 15229465]
25. Lea RA, Dickson S, Benowitz NL. Within-subject variation of the salivary 3HC/COT ratio in regular daily smokers: prospects for estimating CYP2A6 enzyme activity in large-scale surveys of nicotine metabolic rate. Journal of analytical toxicology. 2006; 30(6):386–9. [PubMed: 16872570]
26. Mooney ME, Li ZZ, Murphy SE, Pentel PR, Le C, Hatsukami DK. Stability of the nicotine metabolite ratio in ad libitum and reducing smokers. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2008; 17(6):1396–400.

27. Binnington MJ, Zhu AZ, Renner CC, Lanier AP, Hatsukami DK, Benowitz NL, et al. CYP2A6 and CYP2B6 genetic variation and its association with nicotine metabolism in South Western Alaska Native people. Pharmacogenetics and genomics. 2012; 22(6):429–40. [PubMed: 22569203]
28. Khuder SA. Effect of cigarette smoking on major histological types of lung cancer: a meta-analysis. Lung cancer. 2001; 31(2–3):139–48. [PubMed: 11165392]
29. Wassenaar CA, Ye Y, Cai Q, Aldrich MC, Knight J, Spitz MR, et al. CYP2A6 Reduced Activity Gene Variants Confer Reduction in Lung Cancer Risk in African American Smokers - Findings from Two Independent Populations. Carcinogenesis. 2014
30. Wong HL, Murphy SE, Hecht SS. Cytochrome P450 2A-catalyzed metabolic activation of structurally similar carcinogenic nitrosamines: N′-nitrosonornicotine enantiomers, N-nitrosopiperidine, and N-nitrosopyrrolidine. Chem Res Toxicol. 2005; 18(1):61–9. [PubMed: 15651850]
31. Malaiyandi V, Lerman C, Benowitz NL, Jepson C, Patterson F, Tyndale RF. Impact of CYP2A6 genotype on pretreatment smoking behaviour and nicotine levels from and usage of nicotine replacement therapy. Molecular psychiatry. 2006; 11(4):400–9. [PubMed: 16402128]
32. Mwenifumbo JC, Myers MG, Wall TL, Lin SK, Sellers EM, Tyndale RF. Ethnic variation in CYP2A6*7, CYP2A6*8 and CYP2A6*10 as assessed with a novel haplotyping method. Pharmacogenetics and genomics. 2005; 15(3):189–92. [PubMed: 15861044]
33. Nakajima M, Fukami T, Yamanaka H, Higashi E, Sakai H, Yoshida R, et al. Comprehensive evaluation of variability in nicotine metabolism and CYP2A6 polymorphic alleles in four ethnic populations. Clinical pharmacology and therapeutics. 2006; 80(3):282–97. [PubMed: 16952495]
34. Benowitz NL, Perez-Stable EJ, Herrera B, Jacob P 3rd. Slower metabolism and reduced intake of nicotine from cigarette smoking in Chinese-Americans. Journal of the National Cancer Institute. 2002; 94(2):108–15. [PubMed: 11792749]
35. Perez-Stable EJ, Herrera B, Jacob P 3rd, Benowitz NL. Nicotine metabolism and intake in black and white smokers. JAMA. 1998; 280(2):152–6. [PubMed: 9669788]
36. Slattery ML, Schumacher MC, Lanier AP, Edwards S, Edwards R, Murtaugh MA, et al. A prospective cohort of American Indian and Alaska Native people: study design, methods, and implementation. American journal of epidemiology. 2007; 166(5):606–15. [PubMed: 17586578]
37. Howard BV, Roman MJ, Devereux RB, Fleg JL, Galloway JM, Henderson JA, et al. Effect of lower targets for blood pressure and LDL cholesterol on atherosclerosis in diabetes: the SANDS randomized trial. Jama. 2008; 299(14):1678–89. [PubMed: 18398080]
38. Sonneborn, L. Chronology of American Indian history. New York: Facts On File; 2007. Updated ed
39. Tanner JA, Novalen M, Jatlow P, Huestis MA, Murphy SE, Kaprio J, et al. Nicotine Metabolite Ratio (3-hydroxycotinine/cotinine) in Plasma and Urine by Different Analytical Methods and Laboratories: Implications for Clinical Implementation. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2015
40. St Helen G, Novalen M, Heitjan DF, Dempsey D, Jacob P 3rd, Aziziyeh A, et al. Reproducibility of the nicotine metabolite ratio in cigarette smokers. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2012; 21(7):1105–14.
41. St Helen G, Jacob P 3rd, Benowitz NL. Stability of the nicotine metabolite ratio in smokers of progressively reduced nicotine content cigarettes. Nicotine & tobacco research : official journal of the Society for Research on Nicotine and Tobacco. 2013; 15(11):1939–42. [PubMed: 23674838]
42. Wassenaar CA, Zhou Q, Tyndale RF. CYP2A6 genotyping methods and strategies using real-time and end point PCR platforms. Pharmacogenomics. 2016; 17(2):147–62. [PubMed: 26670214]
43. Al Koudsi N, Ahluwalia JS, Lin SK, Sellers EM, Tyndale RF. A novel CYP2A6 allele (CYP2A6*35) resulting in an amino-acid substitution (Asn438Tyr) is associated with lower CYP2A6 activity in vivo. Pharmacogenomics J. 2009; 9(4):274–82. [PubMed: 19365400]
44. Xu C, Rao YS, Xu B, Hoffmann E, Jones J, Sellers EM, et al. An in vivo pilot study characterizing the new CYP2A6*7, *8, and *10 alleles. Biochem Biophys Res Commun. 2002; 290(1):318–24. [PubMed: 11779172]

45. Mwenifumbo JC, Lessov-Schlaggar CN, Zhou Q, Krasnow RE, Swan GE, Benowitz NL, et al. Identification of novel CYP2A6*1B variants: the CYP2A6*1B allele is associated with faster in vivo nicotine metabolism. Clinical pharmacology and therapeutics. 2008; 83(1):115–21. [PubMed: 17522595]

46. Chenoweth MJ, O'Loughlin J, Sylvestre MP, Tyndale RF. CYP2A6 slow nicotine metabolism is associated with increased quitting by adolescent smokers. Pharmacogenetics and genomics. 2013; 23(4):232–5. [PubMed: 23462429]

47. Al Koudsi N, Hoffmann EB, Assadzadeh A, Tyndale RF. Hepatic CYP2A6 levels and nicotine metabolism: impact of genetic, physiological, environmental, and epigenetic factors. European journal of clinical pharmacology. 2010; 66(3):239–51. [PubMed: 20012030]

48. Lerman C, Tyndale R, Patterson F, Wileyto EP, Shields PG, Pinto A, et al. Nicotine metabolite ratio predicts efficacy of transdermal nicotine for smoking cessation. Clinical pharmacology and therapeutics. 2006; 79(6):600–8. [PubMed: 16765148]

49. Tanner JA, Chenoweth MJ, Tyndale RF. Pharmacogenetics of nicotine and associated smoking behaviors. Curr Top Behav Neurosci. 2015; 23:37–86. [PubMed: 25655887]

50. Raghavan M, Steinrucken M, Harris K, Schiffels S, Rasmussen S, DeGiorgio M, et al. POPULATION GENETICS. Genomic evidence for the Pleistocene and recent population history of Native Americans. Science. 2015; 349(6250):aab3884. [PubMed: 26198033]

51. Templeton AR. The reality and importance of founder speciation in evolution. Bioessays. 2008; 30(5):470–9. [PubMed: 18404703]

52. Bamshad M, Wooding SP. Signatures of natural selection in the human genome. Nat Rev Genet. 2003; 4(2):99–111. [PubMed: 12560807]

53. Fumagalli M, Sironi M. Human genome variability, natural selection and infectious diseases. Curr Opin Immunol. 2014; 30:9–16. [PubMed: 24880709]

54. Mwenifumbo JC, Tyndale RF. Genetic variability in CYP2A6 and the pharmacokinetics of nicotine. Pharmacogenomics. 2007; 8(10):1385–402. [PubMed: 17979512]

55. Wang J, Pitarque M, Ingelman-Sundberg M. 3′-UTR polymorphism in the human CYP2A6 gene affects mRNA stability and enzyme expression. Biochem Biophys Res Commun. 2006; 340(2): 491–7. [PubMed: 16378601]

56. Pitarque M, Rodriguez-Antona C, Oscarson M, Ingelman-Sundberg M. Transcriptional regulation of the human CYP2A6 gene. The Journal of pharmacology and experimental therapeutics. 2005; 313(2):814–22. [PubMed: 15671201]

57. Hakooz N, Hamdan I. Effects of dietary broccoli on human in vivo caffeine metabolism: a pilot study on a group of Jordanian volunteers. Curr Drug Metab. 2007; 8(1):9–15. [PubMed: 17266520]

58. Rae JM, Johnson MD, Lippman ME, Flockhart DA. Rifampin is a selective, pleiotropic inducer of drug metabolism genes in human hepatocytes: studies with cDNA and oligonucleotide expression arrays. The Journal of pharmacology and experimental therapeutics. 2001; 299(3):849–57. [PubMed: 11714868]

59. Itoh M, Nakajima M, Higashi E, Yoshida R, Nagata K, Yamazoe Y, et al. Induction of human CYP2A6 is mediated by the pregnane X receptor with peroxisome proliferator-activated receptor-gamma coactivator 1alpha. The Journal of pharmacology and experimental therapeutics. 2006; 319(2):693–702. [PubMed: 16857725]

60. Ross KC, Gubner NR, Tyndale RF, Hawk LW Jr, Lerman C, George TP, et al. Racial differences in the relationship between rate of nicotine metabolism and nicotine intake from cigarette smoking. Pharmacology, biochemistry, and behavior. 2016; 148:1–7.

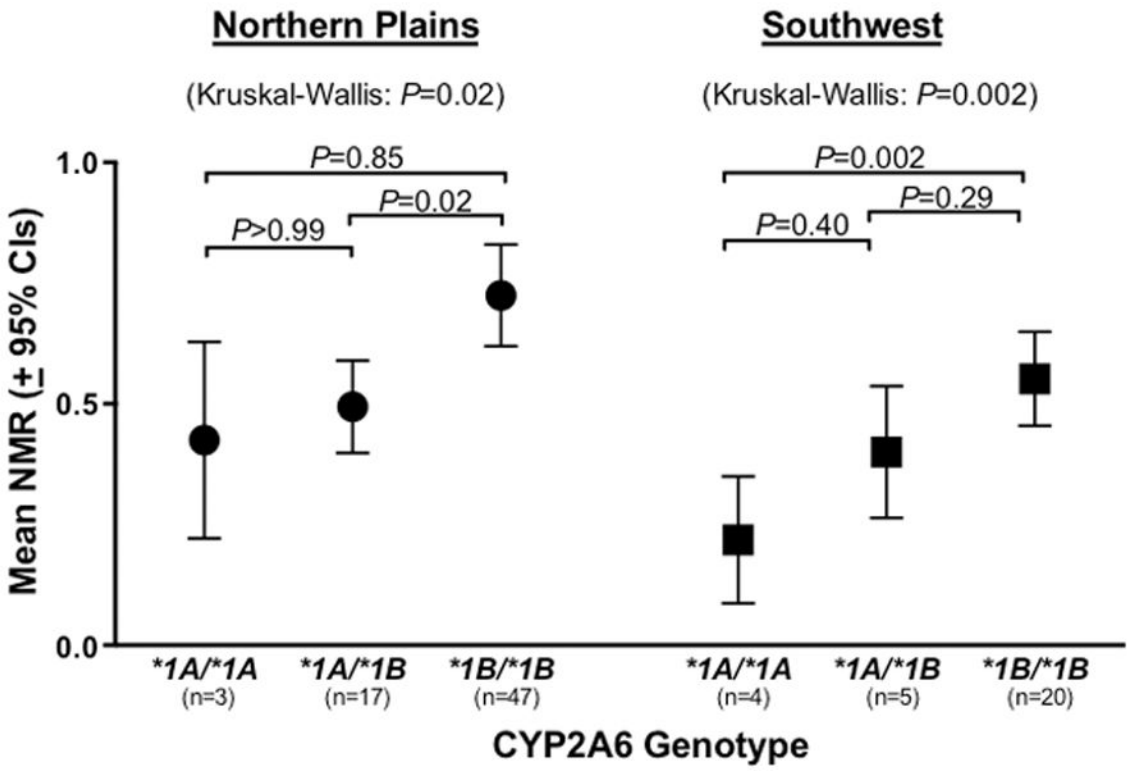

Figure 1.

Comparison of CYP2A6 activity (NMR) between the wild-type *CYP2A6* genotype groups **1A/*1A*, **1A/*1B*, and **1B/*1B*. Individuals included in this analysis do not possess any other tested variants. *P* values for multiple group comparisons (across all three genotypes) are based on Kruskal-Wallis tests. *P* values comparing between groups are based on Dunn's Multiple Comparisons tests. One outlier, 1.5 standard deviations from the mean, was excluded from the plot for illustration purposes, but was included in the statistical analyses (was one of four members of the Northern Plains **1A*1A* group, NMR=1.37).

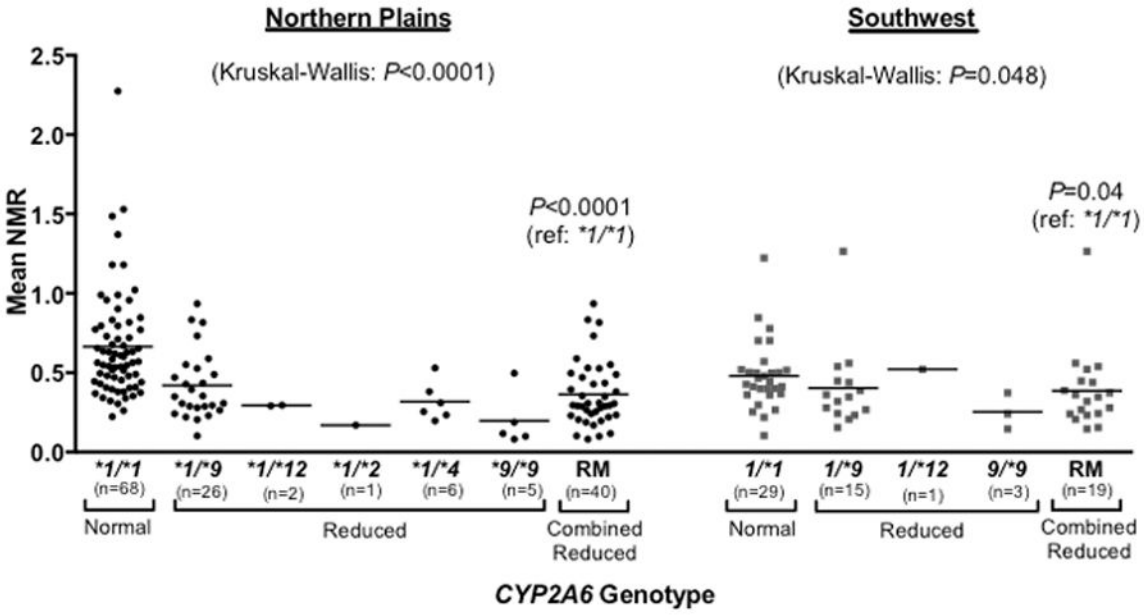

Figure 2.

Association between *CYP2A6* genotype and CYP2A6 activity (NMR) among smokers. The **1/*1* group represents the normal metabolizers (those who do not possess any known CYP2A6 decrease- or loss-of-function alleles; for simplicity the wild-type **1A* and **1B* alleles were assessed generically as **1* for these analyses). The reduced metabolizer group (RM) combines all individuals who possess one or more decrease- or loss-of-function alleles (i.e. the following genotypes: **1/*2*, **1/*4*, **1/*9*, **9/*9,* and **1/*12*). *P* values for multiple group comparisons (across all genotypes) are based on Kruskal-Wallis tests. *P* values comparing **1/*1* and RM groups are based on Mann-Whitney tests.

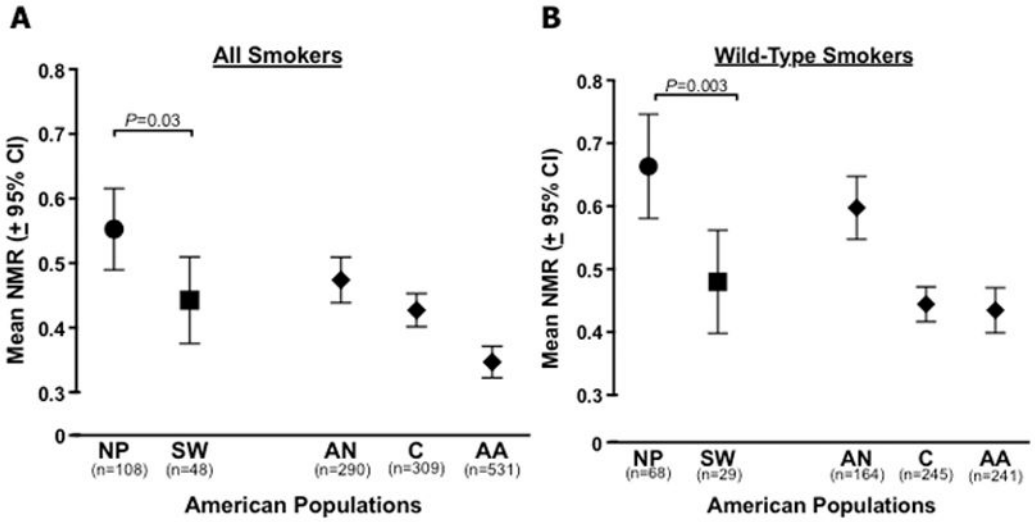

Figure 3.

Comparison of CYP2A6 activity (NMR) among the two tribal populations of smokers (NP, Northern Plains; SW, Southwest). (A) All smokers included in analyses, regardless of *CYP2A6* genotype. (B) Smokers with wild-type genotypes; **1A/*1A*, **1A/*1B*, and **1B/*1B* genotypes only (*CYP2A6* reduced metabolizer (RM) genetic variants were excluded). *P* values are based on Mann-Whitney tests. Other American smoking populations included for visual comparison (AN, Alaska Native; C, Caucasian; AA, African American). *CYP2A6* genotype and NMR data from other American populations taken from the following sources: AN [27]; C [48]; AA [18].

Table 1

Comparison of Northern Plains (n=318[a]) and Southwestern (n=172[a]) *CYP2A6* allele frequencies, and a summary of frequencies from previously studied populations of different ethnic backgrounds

CYP2A6 Allele	Activity	Allele Frequency (%)								
		Northern Plains	Southwest	*P* value [b]	Alaska Native	Caucasian	African American	Japanese	Chinese	Korean
**1B*	Increase	**69.7**	**61.6**	**0.01**	65.3	26.7–35.0	11.2–18.2	25.6–54.6	40.6–51.3	37.1–57.0
**2*	Inactive	**0.3**	**0.6**	0.53	0.4	1.1–5.3	0–1.1	0	0	0
**4*	Inactive	**1.6**	**0.3**	0.07	14.5	0.13–4.2	0.5–2.7	17.0–24.2	4.9–15.1	10.8–11.0
**7*	Inactive	**0**	**0**	–	0	0–0.3	0	6.3–12.6	2.2–9.8	3.6–9.8
**9*	Decrease	**11.9**	**20.9**	**0.0002**	8.9	5.2–8.0	5.7–9.6	19.0–20.7	15.6–15.7	19.6–22.3
**12*	Decrease	**0.3**	**0.3**	0.95	0.4	0–3.0	0–0.4	0–0.8	0	0
**17*	Inactive	**0**	**0**	–	0	0	7.1–10.5	0	0	0
**35*	Decrease	**0**	**0.3**	0.17	0	0	2.5–2.9	0.8	0.5	–

[a] Only unrelated tribal participants have been included in this analysis. Relatedness was determined based on self-report and genetic analyses

[b] *P* values compare NP and SW allele frequencies and are based on Chi-Squared tests

[c] Allele frequency data for Alaska Native, Caucasian, African American, Japanese, Chinese, and Korean populations taken from Tanner et al. 2015 [49]

[d] Genotype frequencies did not deviate significantly from Hardy-Weinberg equilibrium ($P>0.05$ for each genotypes in each tribe)

Table 2

Linear regression analyses of NMR among Northern Plains and Southwestern smokers

Variable	B	95% CI for B	Beta	Variation in NMR accounted for by each variable [a]	*P* value
a. Both Tribal Populations Combined (n=150 included in model)					
CYP2A6 Genotype [b]	0.18	0.11 to 0.25	0.38	14.52%	**<0.001**
Gender (male=1, female=0)	−0.04	−0.14 to 0.05	−0.07	0.45%	0.37
Age (years)	0.0001	−0.003 to 0.004	0.02	0.03%	0.82
Traditional Tobacco user (yes=1, no=0)	−0.01	−0.12 to 0.10	−0.02	0.03%	0.81
Body Mass Index	−0.008	−0.01 to −0.001	−0.17	1.35%	**0.03**
CPD	0.003	−0.004 to 0.01	0.07	0.45%	0.36
Tribal population (NP=1, SW=2)	−0.10	−0.20 to 0.002	−0.15	2.05%	0.06
b. Northern Plains Smokers (n=108 included in model)					
CYP2A6 Genotype [b]	0.21	0.13 to 0.30	0.45	19.71%	**<0.001**
Body Mass Index	−0.007	−0.02 to 0.001	−0.16	2.40%	0.07
c. Southwestern Smokers (n=48 included in model)					
CYP2A6 Genotype [b]	0.11	0.0001 to 0.22	0.29	8.12%	**0.05**
Body Mass Index	−0.005	−0.02 to 0.005	−0.14	1.99%	0.33

[a] Variation in NMR accounted for by each variable is determined by: (Part Correlation)^2 x 100

[b] *CYP2A6* genotype was coded as 0 for individuals with no known CYP2A6 reduced activity alleles (i.e. **1A/*1A*, **1A/*1B*, **1B/*1B* genotypes), −1 for those possessing one decrease-of-function allele (i.e. **1/*9* and **1/*12* genotypes), and −2 for those possessing one or more loss-of-function or two decrease-of-function alleles (i.e. **1/*2*, **1/*4*, and **9/*9* genotypes)

[c] Both tribal populations combined: R^2=0.222, *P*<0.001. R^2 indicates the proportion of variance in NMR levels (22.2%) that is explained by this model.

[d] NP smokers: R^2=0.231, *P*<0.001. R^2 indicates the proportion of variance in NMR levels (23.1%) that is explained by this model.

[e] SW smokers: R^2=0.092, *P*=0.11. R^2 indicates the proportion of variance in NMR levels (9.2%) that is explained by this model

Table 3

Linear regression analyses of NMR among wild-type (**1A/*1A, *1A/*1B, *1B/*1B* genotypes) Northern Plains and Southwestern smokers combined into one sample

Variable	B	95% CI for B	Beta	Variation in NMR accounted for by each variable [a]	*P* value
a. Original Model (n=97)					
*CYP2A6*1B* Genotype [b]	0.14	0.04 to 0.23	0.27	7.34%	**0.006**
Tribal Population (NP=1, SW=2)	−0.17	−0.30 to 0.04	−0.25	6.00%	**0.01**
b. Original Model Plus Interaction Term "Genotype x Tribe" (n=97)					
*CYP2A6*1B* Genotype [b]	0.09	−0.20 to 0.37	0.17	0.32%	0.55
Tribal Population (NP=1, SW=2)	−0.23	−0.56 to 0.11	−0.33	1.64%	0.19
Genotype x Tribe Interaction [c]	0.04	−0.16 to 0.23	0.13	0.12%	0.72

[a] Variation in NMR accounted for by each variable is determined by: (Part Correlation)^2 x 100

[b] *CYP2A6* genotype was coded as 0 for individuals with **1A/*1A* genotype, 1 for those possessing **1A/*1B* genotype, and 2 for those possessing **1B/*1B* genotype.

[c] *CYP2A6*1B* genotype x tribal population interaction term

[d] Original Model: R^2=0.142, *P*=0.001. R^2 indicates the proportion of variance in NMR levels (16.5%) that is explained by this model.

Original Model + Interaction Term: R^2 change=0.001, *P*=0.72. The R^2 change tells you the degree of change in your model with the addition of the interaction term (genotype x tribe).

HHS Public Access

Author manuscript

Pharmacogenet Genomics. Author manuscript; available in PMC 2017 July 01.

Published in final edited form as:

Pharmacogenet Genomics. 2016 July ; 26(7): 340–350. doi:10.1097/FPC.0000000000000222.

Disposition Kinetics and Metabolism of Nicotine and Cotinine in African American smokers: Impact of *CYP2A6* Genetic Variation and Enzymatic Activity

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Abstract

Objective—The rate of nicotine metabolism, determined primarily by CYP2A6 activity, influences tobacco dependence and smoking-induced disease risk. The prevalence of *CYP2A6* gene variants differs by race, with greater numbers in African American (AA) compared to Caucasians. We studied nicotine disposition kinetics and metabolism by *CYP2A6* genotype and enzymatic activity, as measured by nicotine metabolite ratio (NMR), in AA smokers.

Methods—Subjects were administered IV infusions of deuterium-labeled nicotine and cotinine. Plasma and urine concentrations of nicotine and metabolites were measured, and pharmacokinetic parameters estimated.

Results—Pharmacokinetic parameters and urine metabolite excretion data were analyzed by *CYP2A6* genotype and by NMR. A number of gene variants were associated with markedly reduced nicotine and cotinine clearances. NMR was strongly correlated with nicotine (r=0.72) and cotinine (r=0.80) clearances. Subjects with higher NMR excreted significantly greater nicotine C-oxidation and lower non C-oxidation products compared to lower NMR subjects.

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Conflict of Interest/Disclosure

NLB serves as a paid consultant to pharmaceutical companies that are developing or that market smoking cessation medications. He also has been a paid expert witness in litigation against tobacco companies, including on issues related to light cigarettes. RFT has served as paid consultant to pharmaceutical companies on unrelated topics. None of the other authors have any competing interests to declare.

Conclusions—*CYP2A6* genotype, NMR and nicotine pharmacokinetic data may inform studies of individual differences in smoking behavior and biomarkers of nicotine exposure.

Keywords

Nicotine; cotinine; smoking; genetics; CYP2A6; drug metabolism; pharmacokinetics

Introduction

African American smokers differ from non-Hispanic White smokers in several respects. African Americans on average smoke fewer cigarettes per day, take in more nicotine and tobacco smoke per cigarette, are more highly dependent and have a greater risk of lung cancer compared to White smokers [1–4]. They also differ in the average rate of nicotine metabolism [5]. The rate of nicotine metabolism is an important determinant of smoking behavior and its consequences, including the number of cigarettes smoked per day, the ease of quitting smoking and the risk of smoking-induced lung cancer [6, 7].

Nicotine is primarily metabolized by the liver enzyme CYP2A6 to cotinine. Nicotine is also metabolized by glucuronidation (primarily by UGT2B10) and N-oxidation (primarily by FMO3), and there may be small contributions of other enzymes [8]. Cotinine is the major proximal metabolite of nicotine. Cotinine is metabolized exclusively by CYP2A6 to trans-3' hydroxycotinine (3HC), and the ratio of 3HC/cotinine, known as the nicotine metabolite ratio (NMR), is a validated biomarker of CYP2A6 enzymatic activity [9, 10]. Large racial/ethnic differences in the rate and pathways of nicotine and cotinine metabolism, as well as the frequency of *CYP2A6* gene variants, are known to exist [9, 11–13]. An understanding of the relationship between nicotine clearance and metabolism phenotypes and genotypes may be helpful in relating genotype and phenotype to smoking behavior and disease risk.

We previously published a detailed analysis of the relationship between the metabolism and disposition kinetics of nicotine and common variants in the *CYP2A6* gene, but that study was primarily among Caucasian smokers [14]. African Americans possess at reasonable frequencies some of the same alleles as Caucasians, such as *CYP2A6*9*, but also a number of different *CYP2A6* gene variants, such as *CYP2A6*17, *20, *23–*27* and **35*, which are generally not found in Caucasians. These various *CYP2A6* gene variants are associated with reduced NMR but have not been examined with respect to nicotine pharmacokinetics [15–17]. Given the underlying racial/ethnic differences in smoking patterns, lung cancer risk and *CYP2A6* gene variants, it is important to assess how these *CYP2A6* gene variants impact nicotine clearance. Thus, we performed the first investigation of *CYP2A6* genetic variation in association with nicotine pharmacokinetics and metabolism among AA smokers. The nicotine metabolite ratio has been assessed by quartiles in several clinical trials relating NMR to smoking cessation in response to pharmacotherapy [18–21]. Another aim of our study was to examine NMR quartiles in association with nicotine pharmacokinetics and metabolism in AA smokers.

Methods

Subjects

Seventy healthy African American smokers of 5 or more cigarettes per day were recruited and 60 completed the pharmacokinetic study. They were aged between 21 and 60 years (average 35) and smoked an average of 13.7 cigarettes per day (range 5 – 35). Participants were recruited via Craigslist and newspaper advertisements. They were screened for study participation by telephone. Exclusion criteria included pregnancy, use of known drug metabolism altering medications, uncontrolled hypertension or diabetes, heart, lung and cardiovascular disease, cancer, liver and kidney disease, and active substance abuse or dependence. The study was approved by the Institutional Review Boards at the University of California San Francisco and the University of Toronto.

Experimental Procedure

Subjects were asked to come to the Clinical Study Center at the San Francisco General Hospital for a four day pharmacokinetic study after 7 days at home during which they smoked their usual brand of cigarettes, which were supplied by the study. During these 7 days subjects smoked an average of 12.2 cigarettes per day (range 4–29). They came to the hospital the evening before and abstained from cigarettes starting at 10 PM on the night prior to infusion.

In a fasting state at about 9:00 AM, subjects received a simultaneous thirty minute infusion of deuterium-labeled nicotine-d2 (3',3 '-dideuteronicotine) and cotinine-d4 (2, 4, 5, 6-tetradeuterocotinine). The doses of nicotine and cotinine were 2.0 ug/kg/min. The synthesis of deuterium labeled compounds has been described previously [22]. Blood samples for measurement of plasma nicotine-d2 concentrations were collected at 0, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360 and 480 minutes, and then at 12, 16, 24, 48, 72 hours after infusion. We also measured cotinine and 3HC levels (unlabeled, d2 and d4) in the 360 minute plasma sample for determination of the plasma 3HC/cotinine ratio (NMR).

Genotyping of CYP variants was performed according to previously described protocols. Participants were genotyped for *CYP2A6* reduced/null activity alleles predominantly found in African populations, *CYP2A6*17*, **20*, **23-*28*, **31*, **35*, and for those also found in other racial populations, *CYP2A6*2*, **4*, **9* and **12* [15, 23–27]. No participants with **12, *20, *23,* or **28* alleles were identified.

Chemical analysis of nicotine and metabolites

Nicotine and cotinine concentrations in plasma were determined by gas chromatography-mass spectrometry[28]. Plasma concentration of 3HC and urine concentrations of nicotine, cotinine, 3HC and nicotine N-oxide were measured by liquid chromatography – tandem mass spectrometry[10]. Concentrations of nicotine, cotinine, and 3HC glucuronides in urine were measured as the difference in analyte concentrations after and before enzymatic hydrolysis, as described previously[29]. The limits of quantitation for nicotine, cotinine and 3HC in plasma were 0.1 ng/ml. For urine analytes, the limits of quantitation were 10 ng/ml

for nicotine, nicotine N-oxide, cotinine and 3HC and 1 ng/ml for minor species (nornicotine, cotinine N-oxide, norcotinine).

Pharmacokinetic analysis

Pharmacokinetic parameters were estimated from blood concentration and urinary nicotine and metabolite data by use of model independent methods, as described previously using Phoenix WinNonlin 6.3, Pharsight, Mountain View, CA [14].

Half-life was computed by least squares linear regression. We computed nicotine clearance as:

$$CL_{NIC}=[Dose_{NIC-d2}]/[AUC_{NIC-d2}],$$

where, Dose is the dose of nicotine-d2 infused and AUC is the area under the plasma nicotine-d2 concentration time curve extrapolated to infinity. Cotinine clearance was estimated the same way using cotinine-d4 dose and AUC. Fractional conversion of nicotine to cotinine (*f*) was estimated by use of blood levels of cotinine-d2 generated from infused nicotine-d2 and the clearance of cotinine, determined by infusion of cotinine-d4 as follows:

$$f=[AUC_{COT-d2}/Dose_{NIC-d2}] \times CL_{COT-d4}.$$

The metabolic clearance of nicotine by way of the cotinine pathway was computed as $[CL_{NIC\text{-}d2}] \times f$. Because metabolism of nicotine to cotinine is primarily mediated by CYP2A6, this measure is believed to reflect CYP2A6 enzymatic activity.

At steady state the ratio of CLCOT/f is the factor (K) that converts plasma cotinine concentration to daily intake of nicotine derived from tobacco use as follows: $D_{NIC} = [P_{COT}] \times [CL_{COT}/f] = P_{COT} \times K$, where DNIC is the daily intake of nicotine from smoking (mg/24 hr) and PCOT is steady state or time-weighted average plasma cotinine (ng/ml)[22]. We computed this conversion factor and used the baseline plasma cotinine value, obtained during ad libitum smoking prior to study entry, to estimate daily intake of nicotine from each subject.

As mentioned previously, the plasma NMR was measured as a biomarker of CYP2A6 enzymatic activity. The NMR based on d2-labeled compounds measured at 360 min was used to group subjects into quartiles of enzymatic activity. We used the 360 min NMR to reduce the intersubject variability that may be seen with the NMR based on natural nicotine, which may be influenced by recency of smoking. We also did a similar analysis using quartiles based on unlabeled metabolites at 360 min to assess possible bias in using the labeled ratios. The results were nearly identical.

Urine metabolites were analyzed as a molar percentage of the sum of all labeled nicotine metabolites excreted within an eight hour collection, as well as ratios of sequential metabolites to the parent. In addition, we examined the molar sum of all metabolites formed by C-oxidation (cotinine + 3HC + their respective glucuronides, cotinine N-oxide,

nornicotine and norcotinine) and the sum of nicotine and metabolites generated via pathways other than by C-oxidation (nicotine + nicotine glucuronide and nicotine N-oxide) as a fraction of the total of all nicotine and metabolites excreted in urine.

Statistical analysis

Descriptive statistics were computed for pharmacokinetic parameters by genotype and by NMR quartile. Pharmacokinetic parameter means were compared using one-way ANOVA models, where group was the independent variable and each pharmacokinetic parameter was the dependent variable in separate models. Multiple comparisons were made between the four quartile groups, and p-values were adjusted by Tukey's method. The nonparametric Kruskal-Wallis analysis was also used to assess differences in pharmacokinetic parameters across quartiles (data not shown). The significance of the Kruskal-Wallis analysis concurred with the overall significance of the corresponding ANOVA. Pearson correlation coefficients were transformed using Fisher's z transformation to estimate population correlations between NMR from labeled cotinine vs natural cotinine, and NMR with various pharmacokinetic parameters. All analyses were carried out using SAS v. 9.3 (SAS Institute, Inc. Cary, NC, USA). Statistical tests were considered significant at $\alpha = 0.05$.

Results

Genotype frequency and phenotype classification

The frequency of *CYP2A6* genotypes and the phenotype-determined CYP2A6 enzymatic activity estimates (baseline NMR) are shown in table 1. The most prevalent was **1/*1* (62%) followed by **1/*17* (14%), **1/*35* (7%) and **1/**9 and **1/*4H* (3.7% each).

Nicotine and cotinine disposition kinetics

Total clearances and half-lives of nicotine and cotinine, clearance of nicotine via the cotinine pathway and the cotinine-d4 derived 3HC/cotinine ratios are shown by genotype in table 1 and figure 1. As expected there was considerable overlap between genotype groups, with the lowest clearance and NMR values in the *CYP2A6* variant/variant subjects[15]. The lowest CYP2A6 activity as determined by the clearance of nicotine via the cotinine pathway and the NMR were seen in subjects with **9/*26* and **4/*17* genotypes.

Nicotine and Cotinine Disposition Kinetics by NMR Quartiles

The disposition kinetic profiles for nicotine and cotinine according to NMR quartiles are shown in table 2 . In general nicotine and cotinine clearances increased and half-lives decreased with increasing NMR quartile. Statistical differences were consistently found comparing the first and fourth quartiles, with some but fewer statistical differences between second or third vs other quartiles. Pharmacokinetic parameters by NMR quartile are shown in Figures 2 and 3.

Relationship between Plasma Nicotine Metabolite Ratio and Pharmacokinetic Parameters

The NMR at 6 hr based on d4- and d2-labeled compounds were highly correlated (r=0.91) with nearly identical values (ratio 0.98, 95% C.I. 0.91 – 1.06). The d2-NMR was highly

correlated with (r=0.88) but significantly lower than the NMR based on metabolites generated from tobacco use (natural or d0-nicotine;ratio 0.60, 95% CI 0.48-0.72). Pearson correlation with Fisher's z transformation between labeled and natural NMR values, both unlogged and logged, and various pharmacokinetic parameters were examined (Table 3). The log of NMR is theoretically more closely related to clearances than non-logged values [30]. The log NMR (d2) was strongly correlated with total and non-renal clearance of nicotine (r = 0.62), clearance of nicotine to cotinine (r=0.66), total and non-renal clearance of cotinine (r = 0.79 and 0.82, respectively) and inversely correlated with nicotine and cotinine half-lives (r= −0.71 and −0.59, respectively) . The degree of correlations of NMR and the PK variables was similar for unlogged vs logged NMR, and stronger for the d2 or d4-labeled vs unlabeled NMRs.

Impact of CYP2A6 activity on Cotinine as a Biomarker of Daily Nicotine Dose

As described in the methods and in our previous publication, one can use our pharmacokinetic data to compute a conversion factor to estimate daily intake of nicotine from tobacco (and by inference level of tobacco smoke exposure) from the steady state plasma cotinine concentration[22]. Based on our previous research the conversion factor, CL_{COT}/f, generally averages about 0.08 (derived primarily from studies of Caucasian smokers). Thus, a typical person with a cotinine concentration of 200 ng/ml would be taking in 200 × 0.08 or 16 mg nicotine per day. We computed the conversion factor in our subjects, as well as estimated daily nicotine intake derived from the baseline cotinine level and the conversion factor, as shown in table 1. While the average conversion factor ranged from 0.074 to 0.092, we saw some extreme values, ranging from 0.049 to 0.210. The two slowest metabolizers (**4/*17* and **9/*26* genotypes) had the highest conversion factors, meaning that their cotinine levels are much lower than expected for their daily nicotine intake – the result of not converting much nicotine to cotinine. On the other hand, most of the other subjects with reduced function *CYP2A6* variants had lower than normal conversion factors, which would be associated with higher than expected cotinine values, as previously observed among slow metabolizers as a result of reduced cotinine metabolism[31].

Urine metabolites

Eight hour urine metabolite excretion patterns are shown by genotype in table 4. In general subjects with faster metabolism excreted significantly more as total C-oxidation pathway products and less as nicotine plus non-C-oxidation pathway products compared to slower metabolizers. In the two slowest metabolizers (**4/*17* and **9/*26* genotypes) only 11–13% of total metabolites were generated via C-oxidation pathways and 87–89% excreted as nicotine or non-C-oxidation metabolites. In one subject nicotine and nicotine glucuronide accounted for 64% and in the other nicotine and nicotine N-oxide accounted for 58% of total metabolites. Table 5 shows urine metabolite data by NMR quartile. With increasing NMR quartile there were significant increases in 3HC and decreases in nicotine-N-oxide excretion, as well as increases in C-oxidation and decreases in non C-oxidation product excretion. Table 6 shows correlations between various NMR measures and urine metabolite excretion and urine metabolite ratios. Of note, there were no significant correlations between NMR and Nic-gluc/Nic, Cot-gluc/Cot or 3HC-gluc/3HC, suggesting no interaction between NMR and glucuronidation activity.

Discussion

We present novel data on the disposition kinetics and metabolism of nicotine and cotinine in relation to *CYP2A6* genotype and NMR-determined CYP2A6 activity in African American smokers. We have published previously on similar associations in a predominantly Caucasian population [14]. African Americans have a higher prevalence of low frequency reduced and loss of function *CYP2A6* alleles, and slower nicotine and cotinine metabolism on average compared to Caucasians [5, 13]. The present study allows us to examine the functional significance of having a number of *CYP2A6* genetic variants found primarily in African Americans, as well as NMR, on nicotine and cotinine kinetics and metabolism.

The most prevalent minor allele in our subjects was the **17* variant which represents a nonsynonymous SNP in the coding region of the *CYP2A6* gene [32, 33]. Based on in vitro studies the ** 17* variant has substantially reduced enzymatic activity towards nicotine (40–47% activity remaining) consistent with in vivo activity where the **1/*17* and **17/*17* genotypes have 46–61% and 13–16% activity of those with **1/*1* normal metabolism [15, 17, 33]. In the current study, the **17* heterozygotes had only modestly and not significantly reduced nicotine and cotinine clearances compared to wild type. It may be that the wide variation within a genotype group, and the relatively small numbers of subjects with the **17* variant (n=8) genotype in the current pharmacokinetic study relative to the previous studies (N=13–54) contributed to this difference. The **17* variant in combination with other reduced functional alleles (i.e. compound heterozygotes with the **4, *31, *35* alleles) were observed to result in markedly reduced CYP2A6 activity.

The next most prevalent minor alleles in our subjects were **9, *4* and **35.* The **9* allele, which is quite common in both African Americans and Caucasians, is characterized by a SNP in the TATA box of the promoter region which decreases transcription; it is associated in vivo with moderately reduced CYP2A6 activity (**1/*9* have 69–74% activity remaining). The **4* variant represents a gene deletion and is associated with null activity [15, 17]. The **35* variant contains a nonsynonymous SNP in the coding region resulting in decreased nicotine C-oxidation and thermal stability in vitro, and is associated with reduced activity in vivo[34]. Based on the NMR, subjects with the **35* variant were part of group 3 while the **1/*4* heterozygotes were part of group 2. The subject with the **4/*17* variant showed virtually no CYP2A6 activity consistent with **4* being a null allele. The other variant present in two subjects was **26.* This variant represents a nonsynonymous SNP in the coding region and is associated with markedly reduced activity in vitro and in vivo (i.e. **1/*26* had 54–55% activity remaining) [15, 17], consistent with the level of activity found in the single ** 1/*26* heterozygote and the subject with the **9/*26* variant who had virtually no CYP2A6 activity, based on the NMR.

The NMR reflects the metabolism of cotinine to 3HC, a pathway thought to be mediated exclusively or nearly exclusively by CYP2A6 [9]. The nicotine metabolite ratio can be conveniently measured in plasma, saliva or urine of regular tobacco users. The NMR has been recently proposed as a non-invasive biomarker to personalized pharmacotherapy for smoking cessation [20]. Most studies using NMR has grouped NMR by quartiles [18–20]. Few studies have specifically examined nicotine and cotinine pharmacokinetics and

metabolism by NMR. In the present study we found as expected that nicotine and cotinine clearances increased and half-lives decreased with increasing NMR quartiles. We present quantitative estimates of PK parameters by NMR quartiles that can be used to help explain the effects of NMR on smoking behavior and smoking cessation. The substantial magnitude of difference in nicotine half-life by genotype or NMR that we observed could affect smoking behavior and the time course of withdrawal symptoms; and differences in cotinine half-life could have implications for interpreting cotinine levels at various time intervals after smoking cessation.

As observed by us and other researchers, reduced CYP2A6 activity results in an alteration of urine nicotine metabolite excretion patterns [14, 35, 36]. Reduced CYP2A6 activity resulted in reduced excretion of nicotine C-oxidation products (the sum of cotinine, cotinine glucuronide, 3HC, 3HC glucuronide, cotinine N-oxide, norcotinine and nornicotine) and increased excretion of products generated by other pathways (the sum of nicotine, nicotine glucuronide and nicotine N-oxide). In the two subjects with the lowest level of CYP2A6 activity, 90% of nicotine was recovered as non-C-oxidation metabolites, primarily as nicotine and nicotine N-oxide.

In the present study we assessed the NMR based on deuterium-labeled cotinine measured six hours after infusion, as well as NMR based on non-labelled compounds derived from tobacco use. We confirmed the findings of our previous study that there was a strong correlation between NMR and nicotine clearance, and we also found strong correlations between NMR and cotinine clearance and the half-lives of nicotine and cotinine. Not surprisingly, the NMR derived from labeled nicotine or cotinine exhibited stronger correlations with nicotine and cotinine clearances and half-lives (also derived from labeled nicotine) compared to the NMR derived from nicotine from tobacco use. This is likely due to the concordant timing of the kinetics and NMR (i.e. both would be affected by any transitory impacts) and also because of the standardized time interval between dosing of labeled compounds and measurement of NMR. In the natural situation the time from last dose of tobacco (i.e. nicotine) can be quite variable, especially among lighter smokers. The NMR from labeled compounds is lower than the NMR from tobacco use, because 6 hours in an inadequate period of time to achieve full equilibrium between rates of 3HC formation and elimination.

Zhu et al reported that cotinine levels were disproportionately high for the same nicotine intake in people with reduced CYP2A6 enzymatic activity, as cotinine clearance was reduced to a greater extent than cotinine formation from nicotine[31]; this was also observed among those with slower activity in the current study. In addition, we find that those smokers who are fully null (i.e. have no activity) have markedly reduced cotinine levels as they simply do not make much cotinine. Thus, we demonstrate that the relative changes in cotinine formation versus clearance, and therefore the conversion factor, vary substantially according to the particular genetic variant and possibly due to variability in activity of competing metabolic pathways (e.g. glucuronidation).

In conclusion, we present data on the wide variation in nicotine and cotinine pharmacokinetics and metabolism in African American smokers which is influenced by

genetic variation in *CYP2A6*. We also present data relating NMR quartile to pharmacokinetic data. Smokers in the lowest vs highest NMR quartiles demonstrate substantial differences in nicotine and cotinine pharmacokinetics, which are likely to influence smoking behavior and are important to consider in the interpretation of cotinine as a biomarker of tobacco smoke exposure.

Acknowledgments

We would like to thank Dr. Andy Z.X. Zhu and Taraneh Taghavi for careful review of the manuscript, Sandra Tinetti for research coordination, Ewa Hoffmann for *CYP2A6* genotyping, Olivia Yturralde, Trisha Mao and Lita Ramos for analytical chemistry, Faith Allen for data management and Tyson Douglass for editorial assistance. We appreciate the assistance of the nurses on the Clinical Research Center-Clinical and Translational Science Institute research ward at San Francisco General Hospital.

Sources of Funding:

The research was supported by US Public Health Service grants DA02277, DA 020830 and DA 12393 from the National Institute on Drug Abuse. We acknowledge the support of the Endowed Chair in Addictions for the Department of Psychiatry (R.F. Tyndale), CIHR grant TMH-109787 (R.F. Tyndale), the Campbell Family Mental Health Research Institute of CAMH, the CAMH Foundation, the Canada Foundation for Innovation (#20289 and #16014 to R.F. Tyndale) and the Ontario Ministry of Research and Innovation. Clinical studies were performed at the General Clinical Research Center at San Francisco General Hospital Medical Center with support of grant UL1 RO24131 from the National Institutes of Health/National Center for Research Resources.

References

1. Department of Health and Human Services PHS. Tobacco Use Among US Racial/Ethnic Minority Groups — African Americans, American Indians and Alaska Natives, Asian Americans and Pacific Islanders, and Hispanics: A Report of the Surgeon General: USDHHS, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Promotion, Office of Smoking and Health. Washington DC: Government Printing Office; 1998.
2. Haiman CA, Stram DO, Wilkens LR, Pike MC, Kolonel LN, Henderson BE, et al. Ethnic and racial differences in the smoking-related risk of lung cancer. N Engl J Med. 2006; 354(4):333–42. [PubMed: 16436765]
3. Trinidad DR, Perez-Stable EJ, Emery SL, White MM, Grana RA, Messer KS. Intermittent and light daily smoking across racial/ethnic groups in the United States. Nicotine Tob Res. 2009; 11(2):203–10. [PubMed: 19246433]
4. Siahpush M, Singh GK, Jones PR, Timsina LR. Racial/ethnic and socioeconomic variations in duration of smoking: results from 2003, 2006 and 2007 Tobacco Use Supplement of the Current Population Survey. Journal of public health (Oxford, England). 2010; 32(2):210–8.
5. Perez-Stable EJ, Herrera B, Jacob P 3rd, Benowitz NL. Nicotine metabolism and intake in black and white smokers. Jama. 1998; 280(2):152–6. [PubMed: 9669788]
6. Malaiyandi V, Sellers EM, Tyndale RF. Implications of CYP2A6 genetic variation for smoking behaviors and nicotine dependence. Clin Pharmacol Ther. 2005; 77(3):145–58. [PubMed: 15735609]
7. Wassenaar CA, Dong Q, Wei Q, Amos CI, Spitz MR, Tyndale RF. Relationship Between CYP2A6 and CHRNA5-CHRNA3-CHRNB4 Variation and Smoking Behaviors and Lung Cancer Risk. Journal of the National Cancer Institute. 2011; 103(17):1342–6. [PubMed: 21747048]
8. Hukkanen J, Jacob P 3rd, Benowitz NL. Metabolism and disposition kinetics of nicotine. Pharmacological reviews. 2005; 57(1):79–115. [PubMed: 15734728]
9. St Helen G, Novalen M, Heitjan DF, Dempsey D, Jacob P 3rd, Aziziyeh A, et al. Reproducibility of the nicotine metabolite ratio in cigarette smokers. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2012; 21(7):1105–14.

10. Dempsey D, Tutka P, Jacob P 3rd, Allen F, Schoedel K, Tyndale RF, et al. Nicotine metabolite ratio as an index of cytochrome P450 2A6 metabolic activity. Clinical pharmacology and therapeutics. 2004; 76(1):64–72. [PubMed: 15229465]
11. Zhu AZ, Zhou Q, Cox LS, David SP, Ahluwalia JS, Benowitz NL, et al. Association of CHRNA5-A3-B4 SNP rs2036527 with smoking cessation therapy response in African-American smokers. Clin Pharmacol Ther. 2014; 96(2):256–65. [PubMed: 24733007]
12. Chenoweth MJ, O'Loughlin J, Sylvestre MP, Tyndale RF. CYP2A6 slow nicotine metabolism is associated with increased quitting by adolescent smokers. Pharmacogenetics and genomics. 2013; 23(4):232–5. [PubMed: 23462429]
13. McDonagh EM, Wassenaar C, David SP, Tyndale RF, Altman RB, Whirl-Carrillo M, et al. PharmGKB summary: very important pharmacogene information for cytochrome P-450, family 2, subfamily A, polypeptide 6. Pharmacogenetics and genomics. 2012; 22(9):695–708. [PubMed: 22547082]
14. Benowitz NL, Swan GE, Jacob P 3rd, Lessov-Schlaggar CN, Tyndale RF. CYP2A6 genotype and the metabolism and disposition kinetics of nicotine. Clin Pharmacol Ther. 2006; 80(5):457–67. [PubMed: 17112802]
15. Ho MK, Mwenifumbo JC, Al Koudsi N, Okuyemi KS, Ahluwalia JS, Benowitz NL, et al. Association of nicotine metabolite ratio and CYP2A6 genotype with smoking cessation treatment in African-American light smokers. Clin Pharmacol Ther. 2009; 85(6):635–43. [PubMed: 19279561]
16. Mwenifumbo JC, Tyndale RF. Genetic variability in CYP2A6 and the pharmacokinetics of nicotine. Pharmacogenomics. 2007; 8(10):1385–402. [PubMed: 17979512]
17. Mwenifumbo JC, Al Koudsi N, Ho MK, Zhou Q, Hoffmann EB, Sellers EM, et al. Novel and established CYP2A6 alleles impair in vivo nicotine metabolism in a population of Black African descent. Hum Mutat. 2008; 29(5):679–88. [PubMed: 18360915]
18. Lerman C, Tyndale R, Patterson F, Wileyto EP, Shields PG, Pinto A, et al. Nicotine metabolite ratio predicts efficacy of transdermal nicotine for smoking cessation. Clinical pharmacology and therapeutics. 2006; 79(6):600–8. [PubMed: 16765148]
19. Patterson F, Schnoll RA, Wileyto EP, Pinto A, Epstein LH, Shields PG, et al. Toward personalized therapy for smoking cessation: a randomized placebo-controlled trial of bupropion. Clinical pharmacology and therapeutics. 2008; 84(3):320–5. [PubMed: 18388868]
20. Lerman C, Schnoll RA, Hawk LW Jr, Cinciripini P, George TP, Wileyto EP, et al. Use of the nicotine metabolite ratio as a genetically informed biomarker of response to nicotine patch or varenicline for smoking cessation: a randomised, double-blind placebo-controlled trial. The Lancet Respiratory medicine. 2015; 3(2):131–8. [PubMed: 25588294]
21. Schnoll RA, Patterson F, Wileyto EP, Tyndale RF, Benowitz N, Lerman C. Nicotine metabolic rate predicts successful smoking cessation with transdermal nicotine: a validation study. Pharmacology, biochemistry, and behavior. 2009; 92(1):6–11.
22. Benowitz NL, Jacob P 3rd. Metabolism of nicotine to cotinine studied by a dual stable isotope method. Clin Pharmacol Ther. 1994; 56(5):483–93. [PubMed: 7955812]
23. Piliguian M, Zhu AZ, Zhou Q, Benowitz NL, Ahluwalia JS, Sanderson Cox L, et al. Novel CYP2A6 variants identified in African Americans are associated with slow nicotine metabolism in vitro and in vivo. Pharmacogenetics and genomics. 2014; 24(2):118–28. [PubMed: 24305170]
24. Tang DW, Hello B, Mroziewicz M, Fellows LK, Tyndale RF, Dagher A. Genetic variation in CYP2A6 predicts neural reactivity to smoking cues as measured using fMRI. NeuroImage. 2012; 60(4):2136–43. [PubMed: 22342802]
25. Lerman C, Jepson C, Wileyto EP, Patterson F, Schnoll R, Mroziewicz M, et al. Genetic variation in nicotine metabolism predicts the efficacy of extended-duration transdermal nicotine therapy. Clin Pharmacol Ther. 2010; 87(5):553–7. [PubMed: 20336063]
26. Wassenaar CA, Conti DV, Das S, Chen P, Cook EH, Ratain MJ, et al. UGT1A and UGT2B genetic variation alters nicotine and nitrosamine glucuronidation in european and african american smokers. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2015; 24(1):94–104.

27. Wassenaar CA, Zhou Q, Tyndale RF. CYP2A6 genotyping methods and strategies using real-time and end point PCR platforms. Pharmacogenomics. 2016; 17(2):147–62. [PubMed: 26670214]

28. Jacob P 3rd, Yu L, Wilson M, Benowitz NL. Selected ion monitoring method for determination of nicotine, cotinine and deuterium-labeled analogs: absence of an isotope effect in the clearance of (S)-nicotine-3',3'-d2 in humans. Biol Mass Spectrom. 1991; 20(5):247–52. [PubMed: 1883864]

29. Benowitz NL, Jacob P 3rd, Fong I, Gupta S. Nicotine metabolic profile in man: comparison of cigarette smoking and transdermal nicotine. J Pharmacol Exp Ther. 1994; 268(1):296–303. [PubMed: 8301571]

30. Levi M, Dempsey DA, Benowitz NL, Sheiner LB. Prediction methods for nicotine clearance using cotinine and 3-hydroxy-cotinine spot saliva samples II. Model application. J Pharmacokinet Pharmacodyn. 2007; 34(1):23–34. [PubMed: 17206525]

31. Zhu AZ, Renner CC, Hatsukami DK, Lerman C, Swan GE, Benowitz NL, et al. The ability of plasma cotinine to predict nicotine and carcinogen exposure is altered by differences in CYP2A6: the influence of genetics, race and sex. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2013; (11) In press.

32. Ho MK, Mwenifumbo JC, Zhao B, Gillam EM, Tyndale RF. A novel CYP2A6 allele, CYP2A6*23, impairs enzyme function in vitro and in vivo and decreases smoking in a population of Black-African descent. Pharmacogenetics and genomics. 2008; 18(1):67–75. [PubMed: 18216723]

33. Fukami T, Nakajima M, Yoshida R, Tsuchiya Y, Fujiki Y, Katoh M, et al. A novel polymorphism of human CYP2A6 gene CYP2A6*17 has an amino acid substitution (V365M) that decreases enzymatic activity in vitro and in vivo. Clin Pharmacol Ther. 2004; 76:519–27. [PubMed: 15592323]

34. Al Koudsi N, Ahluwalia JS, Lin SK, Sellers EM, Tyndale RF. A novel CYP2A6 allele (CYP2A6*35) resulting in an amino-acid substitution (Asn438Tyr) is associated with lower CYP2A6 activity in vivo. Pharmacogenomics J. 2009; 9(4):274–82. [PubMed: 19365400]

35. Patel YM, Stram DO, Wilkens LR, Park SS, Henderson BE, Le Marchand L, et al. The Contribution of Common Genetic Variation to Nicotine and Cotinine Glucuronidation in Multiple Ethnic/Racial Populations. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2014

36. Murphy SE, Park SS, Thompson EF, Wilkens LR, Patel Y, Stram DO, et al. Nicotine N-glucuronidation relative to N-oxidation and C-oxidation and UGT2B10 genotype in five ethnic/racial groups. Carcinogenesis. 2014; 35(11):2526–33. [PubMed: 25233931]

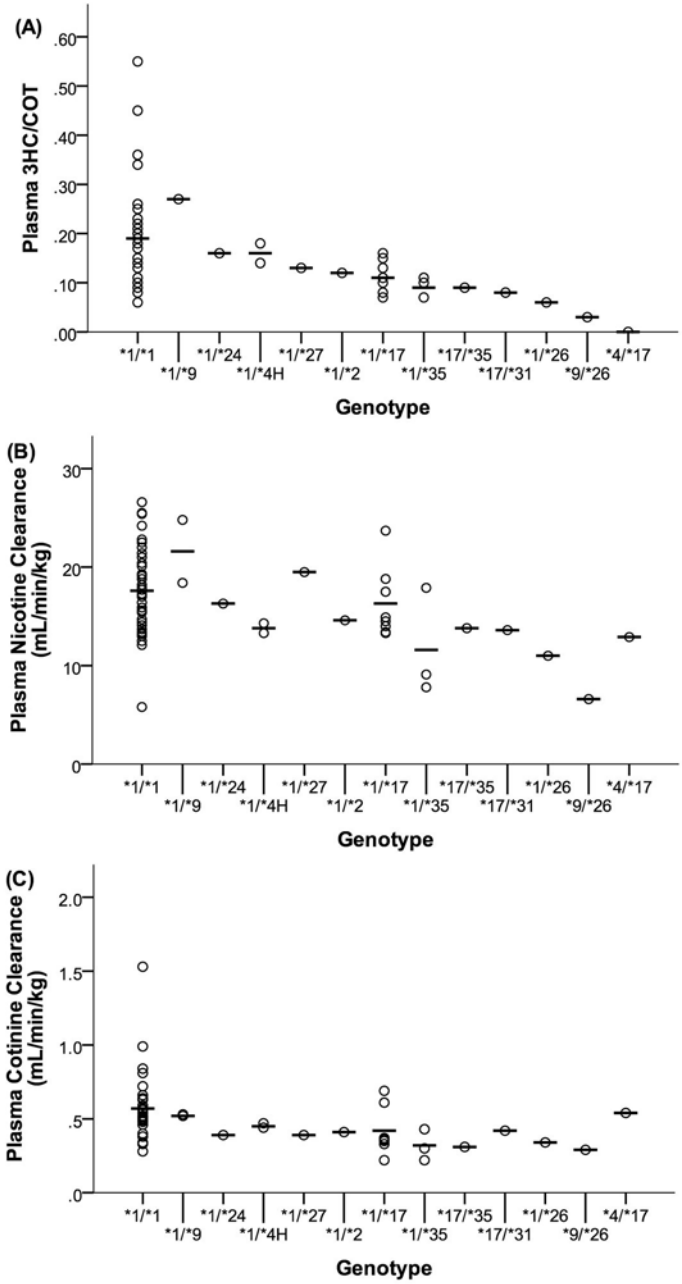

FIGURE 1.

CYP2A6 genotype-phenotype associations for baseline plasma trans-3′-hydroxycotinine/cotinine ratio (3HC/COT) (A), plasma nicotine clearance (B), and plasma cotinine clearance (C).

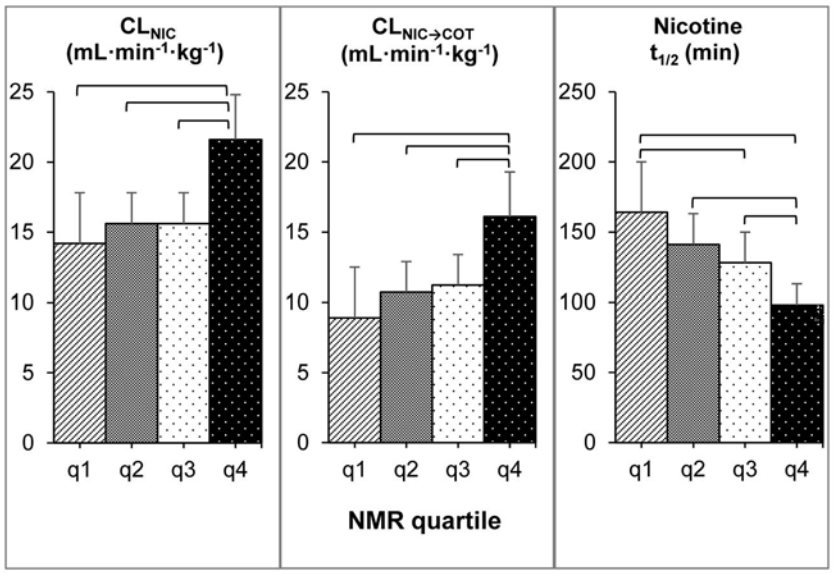

FIGURE 2.

Disposition kinetics of nicotine based on NMR (d2) quartile grouping (mean and SD). Square brackets indicate significant differences between groups ($p<0.05$).

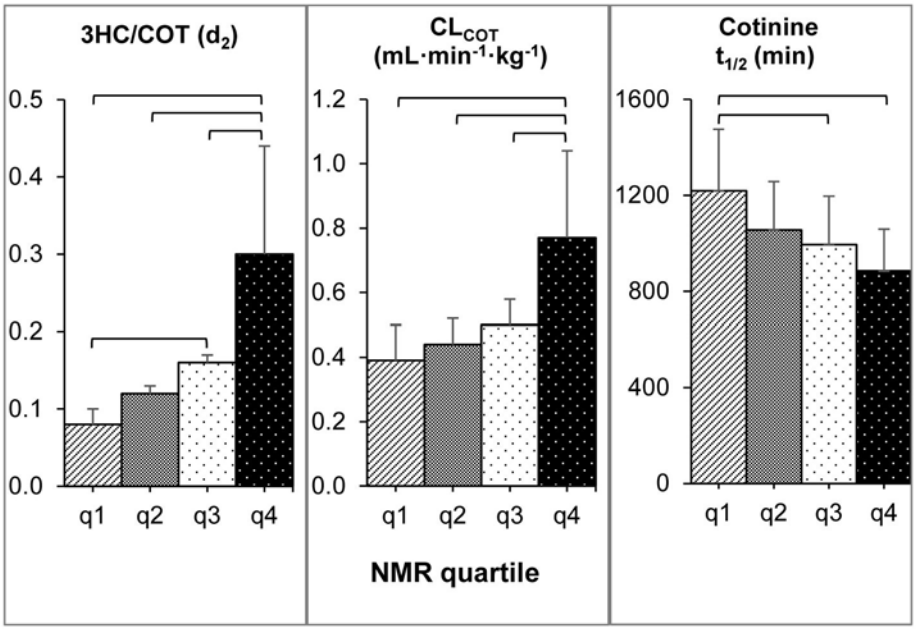

FIGURE 3.

Disposition kinetics of cotinine and 3'-hydroxycotinine/cotinine (3HC/COT-d2) ratio based on 6 hour NMR (d2) quartile grouping (mean and SD). Square brackets indicate significant differences between groups ($p<0.05$).

TABLE 1

CYP2A6 genotype activity, plasma clearance of nicotine and cotinine, and 3HC/COT ratio

CYP2A6 Genotype activity	n	Plasma 3HC/COT baseline†	Plasma 3HCd2/COTd2	CLNIC (mL · min^{-1} · kg^{-1})	CLNIC-COT (mL · min^{-1} · kg^{-1})	Total CLCOT (mL · min^{-1} · kg^{-1})	NIC t1/2 (min)	COT t1/2 (min)	Total CLCOT/f	Baseline COT (ng/mL)	Estimated NIC intake (mg/24h)
*1/*1	37	0.45 (0.27)	0.19 (0.12)	17.6 (4.4)	12.4 (4.2)	0.57 (0.23)	124 (31)	977 (222)	0.092 (0.033)	223.4 (130.6)	20.3 (13.3)
*1/*17	8	0.29 (0.10)	0.12 (0.05)	16.3 (3.6)	10.7 (2.8)	0.42 (0.17)	134 (33)	1107 (131)	0.074 (0.016)	205.7 (60.9)	15.1 (3.4)
*1/*9	2	0.35 (0.21)	0.16	21.6 (4.6)	14.8 (2.3)	0.52 (0.01)	97 (12)	868 (119)	0.079	153.2 (27.5)	11.9 (0.5)
*1/*24	1	0.54	0.15	16.3	13.9	0.39	147	960	0.065	258.7	16.8
*1/*4H	2	0.22 (0.02)	0.14 (0.03)	13.8 (0.7)	8.8 (2.6)	0.45 (0.02)	152 (7)	1197 (128)	0.085 (0.008)	227.7 (22.8)	19.2 (0.2)
*1/*27	1	0.31	0.13	19.5	15.2	0.39	108	1092	0.067	67.3	4.4
*1/*2	1	0.25	0.13	14.6	11.6	0.41	151	1125	0.062	320.3	19.9
*1/*35	3	0.24 (0.06)	0.10 (0.06)	11.6 (5.5)	7.8 (3.1)	0.32 (0.11)	179 (35)	1387 (355)	0.048 (0.011)	211.0 (118.6)	9.4 (4.2)
*17/*35	1	0.25	0.12	13.7	10.7	0.31	167	1164	0.049	196.9	9.7
*17/*35	1	0.17	0.07	13.6	7.9	0.42	158	928	0.065	340.4	22.1
*1/*26	1	0.13	0.07	11.0	6.8	0.34	152	1216	0.059	240.4	14.2
*9/*26	1	0.03	0.18	6.6	2.3	0.29	210	1612	0.122	380.4	46.5
*4/*17	1	0.02	n/a	12.9	3.8	0.54	249	1644	0.210	154.5	32.4
All subjects	60	0.38 (0.25)	0.17 (0.11)	16.6 (4.6)	11.4 (4.2)	0.51 (0.21)	134 (37)	1052 (249)	0.086 (0.035)	221 (114)	18.8 (11.9)

Notes: Values are presented as mean (SD);

† the plasma 3HC/COT ratio from baseline screening sample

TABLE 2

Disposition kinetics of nicotine by nicotine-d2 metabolite ratio (NMR-d2) quartile

	Nicotine-d_2 metabolite ratio (NMR-d_2) quartile					
Variable	**All subjects**	**1st Quartile**	**2nd Quartile**	**3rd Quartile**	**4th Quartile**	**Overall P value**
n	60	17	14	15	14	
Body weight (kg)	80.0 (15.2)	78.6 (14.9)	80.9 (14.3)	82.2 (14.3)	78.3 (18.6)	0.89
A. Nicotine kinetics						
Total CL_{NIC} ($mL \cdot min^{-1} \cdot kg^{-1}$)	16.6 (4.6)	14.2 (3.6)	15.6 (2.2)	15.6 (2.2)	21.6 (3.2)*b,c,d*	<0.001
Renal CL_{NIC} ($mL \cdot min^{-1} \cdot kg^{-1}$)	0.69 (0.59)	0.68 (0.45)	0.73 (0.63)	0.34 (0.63)	1.05 (0.75)*d*	0.01
Nonrenal CL_{NIC} ($mL \cdot min^{-1} \cdot kg^{-1}$)	15.9 (4.5)	13.5 (3.7)	14.9 (2.1)	15.3 (2.1)	20.5 (3.6)*b,c,d*	<0.001
$CL_{NIC \rightarrow COT}$ ($mL \cdot min^{-1} \cdot kg^{-1}$)	11.4 (4.2)	8.9 (2.9)	10.7 (2.3)	11.2 (2.3)	16.1 (4.1)*b,c,d*	<0.001
Fractional conversion, *f*	0.68 (0.11)	0.62 (0.10)	0.68 (0.10)	0.71 (0.10)	0.73 (0.13)	0.053
Nicotine $t_{1/2}$ (min)	134 (37)	164 (36)	141 (22)	128 (22)*a*	98 (15)*b,c,d*	<0.001
Nicotine V_{ss} (L/kg)	2.6 (0.7)	3.0 (0.6)	2.6 (0.7)	2.4 (0.7)	2.4 (0.6)	0.04
B. Cotinine kinetics						
Total CL_{COT} ($mL \cdot min^{-1} \cdot kg^{-1}$)	0.51 (0.21)	0.39 (0.11)	0.44 (0.08)	0.50 (0.08)	0.77 (0.27)*b,c,d*	<0.001
Renal CL_{COT} ($mL \cdot min^{-1} \cdot kg^{-1}$)	0.10 (0.05)	0.10 (0.04)	0.09 (0.05)	0.09 (0.05)	0.13 (0.07)	0.14
Nonrenal CL_{COT} ($mL \cdot min^{-1} \cdot kg^{-1}$)	0.41 (0.19)	0.29 (0.12)	0.35 (0.06)	0.41 (0.06)	0.64 (0.25)*b,c,d*	<0.001
Cotinine $t_{1/2}$ (min)	1053 (249)	1219 (256)	1055 (202)	994 (202)*a*	884 (175)*b*	0.002
Cotinine V_{ss} (L/kg)	0.68 (0.16)	0.64 (0.18)	0.64 (0.13)	0.66 (0.13)	0.85 (0.13)*b,c,d*	<0.001
Plasma 3HC/COT†	0.17 (0.11)	0.08 (0.02)	0.12 (0.01)	0.16 (0.01)*a*	0.30 (0.14)*b,c,d*	<0.001

Notes: NMR-d2: 1st quartile, 0.11; median, 0.14; 3rd quartile, 0.19

a p<0.05, 3rd quartile significantly different from 1st quartile

b p<0.05, 4th quartile significantly different from 1st quartile

c p<0.05, 4th quartile significantly different from 2nd quartile

d p<0.05, 4th quartile significantly different from 3rd quartile

† Plasma 3HC/COT is based on d2 analytes.

TABLE 3

Correlation between NMR and pharmacokinetic parameters

Variable	Pearson Correlation with Fisher's z transformation					
	NMR(d_0)	logNMR(d_0)	NMR(d_2)	logNMR(d_2)	NMR(d_4)	logNMR(d_4)
Total CL_{NIC} ($mL \cdot min^{-1} \cdot kg^{-1}$)	0.52*	0.56*	0.55*	0.62*	0.64*	0.72*
Nonrenal CL_{NIC} ($mL \cdot min^{-1} \cdot kg^{-1}$)	0.51*	0.55*	0.55*	0.62*	0.65*	0.72*
Renal CL_{NIC} ($mL \cdot min^{-1} \cdot kg^{-1}$)	0.14	0.12	0.03	0.04	0.03	0.06
$CL_{NIC \rightarrow COT}$ ($mL \cdot min^{-1} \cdot kg^{-1}$)	0.59*	0.66*	0.59*	0.66*	0.65*	0.73*
Total CL_{COT} ($mL \cdot min^{-1} \cdot kg^{-1}$)	0.75*	0.59*	0.82*	0.79*	0.84*	0.80*
Nonrenal CL_{COT} ($mL \cdot min^{-1} \cdot kg^{-1}$)	0.79*	0.62*	0.84*	0.82*	0.85*	0.82*
Renal CL_{COT} ($mL \cdot min^{-1} \cdot kg^{-1}$)	0.12	0.09	0.23	0.19	0.27	0.20
Nicotine $T_{½}$(min)	−0.65*	−0.74*	−0.62*	−0.71*	−0.70*	−0.76*
Cotinine $T_{½}$ (min)	−0.57*	−0.67*	−0.50*	−0.59*	−0.64*	−0.71*

*$P < 0.001$

TABLE 4

Nicotine and cotinine urine metabolites and metabolite ratios by *CYP2A6* genotype activity

CYP2A6 Genotype activity	n	Nicotine (NIC)	NIC-gluc	Cotinine (COT)	COT-gluc	3HC	3HC-gluc	Nicotine N-oxide (NNO)	Cotinine N-oxide (CNO)	Nornicotine (NNIC)	Norcotinine (NCOT)
*1/*1	37	26.0 (18.4)	4.6 (2.9)	20.0 (7.1)	3.8 (2.6)	15.2 (10.0)	2.7 (2.2)	24.8 (10.0)	1.6 (0.7)	0.7 (0.3)	0.7 (0.3)
*1/*17	8	17.2 (6.7)	4.1 (3.1)	24.2 (3.4)	2.6 (2.6)	13.4 (5.9)	4.5 (4.0)	30.4 (8.1)	1.8 (0.7)	0.8 (0.2)	1.0 (0.3)
*1/*9	2	10.4 (1.0)	5.9 (4.0)	23.0 (5.5)	4.8 (1.0)	16.3 (3.3)	3.3 (1.9)	32.8 (4.7)	1.8 (0.4)	0.9 (0.5)	0.8 (0.1)
*1/*24	1	11.6	1.9	20.5	0.8	25.0	6.2	28.7	3.0	1.0	1.4
*1/*4H	2	28.0 (21.8)	10.3 (2.5)	15.8 (3.3)	5.2 (1.8)	10.6 (7.8)	1.4 (1.5)	24.9 (7.7)	1.7 (0.8)	1.3 (0.8)	0.7 (0.6)
*1/*27	1	39.3	3.2	13.1	2.6	11.6	2.8	24.3	1.7	0.7	0.6
*1/*2	1	7.0	6.3	24.9	4.2	19.7	4.4	26.0	5.3	1.2	1.1
*1/*35	3	35.8 (4.8)	1.7 (0.2)	11.9 (3.6)	0.4 (0.6)	10.3 (6.7)	2.5 (2.6)	34.6 (10.2)	1.4 (0.4)	0.8 (0.2)	0.6 (0.3)
*17/*35	1	4.0	9.0	11.2	4.7	19.5	5.4	40.6	3.2	0.9	1.5
*17/*31	1	47.6	1.6	15.1	1.0	4.1	0.5	27.3	1.6	0.7	0.3
*1/*26	1	36.2	0.9	22.8	0.1	4.0	0.8	33.0	0.6	1.1	0.6
*9/*26	1	15.5	15.7	7.8	1.6	0.8	0.0	57.9	0.1	0.5	0.1
*4/*17	1	45.0	19.1	6.8	2.5	0.0	0.0	23.0	0.0	3.0	0.4
All subjects	60	24.9 (16.8)	5.0 (3.9)	19.5 (6.9)	3.3 (2.5)	14.0 (9.1)	2.9 (2.5)	27.4 (10.1)	1.6 (0.9)	0.8 (0.4)	0.7 (0.3)

CYP2A6 Genotype activity	n	NIC-gluc/total NIC	COT-gluc/total COT	3HC-gluc/total 3HC	NIC-gluc/NIC	COT-gluc/COT	3HC-gluc/3HC	3HC/COT	[3HC + 3HC-gluc]/COT	Non C-oxidation	C-oxidation
*1/*1	37	20.4 (15.5)	16.4 (12.0)	14.1 (8.5)	32.1 (36.0)	22.8 (23.8)	17.5 (11.8)	0.83 (0.69)	0.97 (0.77)	55.5 (16.3)	44.5 (16.3)
*1/*17	8	18.3 (13.5)	9.0 (8.5)	22.5 (7.6)	25.7 (22.7)	10.8 (10.7)	30.2 (13.7)	0.56 (0.24)	0.74 (0.36)	51.8 (11.1)	48.2 (11.1)
*1/*9	2	33.8 (14.2)	17.9 (6.6)	16.3 (5.3)	54.6 (33.1)	22.2 (9.8)	19.7 (7.6)	0.71 (0.03)	0.85 (0.02)	49.1 (9.7)	50.9 (9.7)
*1/*24	1	14.0	3.7	19.9	16.3	3.8	24.9	1.22	1.52	42.2	57.8
*1/*4H	2	31.1 (13.3)	24.5 (2.7)	10.4 (4.1)	48.0 (28.6)	32.5 (4.8)	11.8 (5.1)	0.63 (0.36)	0.72 (0.44)	63.2 (16.6)	36.8 (16.6)
*1/*27	1	7.5	16.4	19.2	8.1	19.6	23.7	0.88	1.09	66.9	33.1
*1/*2	1	47.3	14.4	18.3	89.9	16.8	22.4	0.79	0.97	39.2	60.8
*1/*35	3	4.7 (1.0)	2.7 (3.4)	17.2 (7.5)	4.9 (1.1)	2.9 (3.7)	21.4 (11.4)	0.79 (0.38)	0.98 (0.52)	72.1 (13.6)	27.9 (13.6)
*17/*35	1	69.4	29.7	21.8	226.6	42.3	27.9	1.74	2.23	53.5	46.5
*17/*31	1	3.3	6.2	11.0	3.5	6.7	12.3	0.27	0.31	76.6	23.4
*1/*26	1	2.3	0.5	16.0	2.4	0.5	19.1	0.17	0.21	70.0	30.0
*9/*26	1	50.4	17.1	0.0	101.8	20.6	0.0	0.10	0.10	89.1	10.9

CYP2A6 Genotype activity	n	NIC-gluc/total NIC	COT-gluc/total COT	3HC-gluc/total 3HC	NIC-gluc/NIC	COT-gluc/COT	3HC-gluc/3HC	3HC/COT	[3HC + 3HC-gluc]/COT	Non C-oxidation	C-oxidation
*4/*17	1	29.8	26.9	0.0	42.5	36.8		0.00	0.00	87.2	12.8
All subjects	60	21.1 (16.8)	14.8 (11.3)	15.5 (8.3)	35.0 (42.0)	19.9 (20.6)	19.5 (12.1)	0.76 (0.60)	0.90 (0.69)	57.2 (16.2)	42.8 (16.2)

Notes: Non C-oxidation is [NIC + NIC-gluc + NNO]/TNE × 100; C-oxidation is [COT + COT-gluc + 3HC + 3HC-gluc + CNO + NNIC + NCOT]/TNE × 100

TABLE 5

Nicotine and cotinine urine metabolites (A) and nicotine and cotinine urine metabolite ratios (B) by nicotine-d2 metabolite ratio (NMR-d2) quartile

	Nicotine-d_2 metabolite ratio (NMR-d_2) quartile					
Urine metabolite or ratio	**All subjects**	**1st Quartile**	**2nd Quartile**	**3rd Quartile**	**4th Quartile**	**Overall P value**
n	60	17	14	15	14	
A. Percent (%) of nicotine and metabolites recovered						
Nicotine (NIC)	24.9 (16.8)	27.1 (15.0)	28.4 (19.5)	14.8 (8.1)	29.3 (19.8)	0.06
NIC glucuronide	5.0 (3.9)	5.7 (5.1)	5.4 (3.5)	4.6 (3.7)	4.1 (2.7)	0.67
Cotinine (COT)	19.5 (6.9)	18.0 (5.6)	18.6 (7.9)	22.2 (6.7)	19.1 (7.5)	0.35
COT glucuronide	3.3 (2.5)	2.3 (2.2)	3.6 (2.5)	3.1 (2.0)	4.5 (3.1)	0.11
3-Hydroxycotinine (3HC)	14.0 (9.1)	8.5 (5.2)	11.5 (6.7)	16.7 (6.6)[a]	20.1 (12.4)[b,c]	<0.001
3HC glucuronide	2.9 (2.5)	1.8 (1.7)	2.2 (1.7)	4.3 (3.3)[a]	3.4 (2.4)	0.02
Nicotine N-oxide (NNO)	27.4 (10.1)	33.5 (7.9)	27.1 (7.6)	30.6 (11.1)	16.8 (4.1)[b,c,d]	<0.001
Cotinine N-oxide (CNO)	1.6 (0.9)	1.5 (0.8)	1.7 (1.3)	1.9 (0.7)	1.4 (0.7)	0.45
Nornicotine (NNIC)	0.8 (0.4)	1.0 (0.6)	0.8 (0.3)	0.8 (0.4)	0.5 (0.2)	0.07
Norcotinine (NCOT)	0.7 (0.3)	0.7 (0.3)	0.7 (0.4)	0.9 (0.3)	0.7 (0.3)	0.13
B. Nicotine and cotinine urine metabolite ratios						
NIC-gluc/total NIC × 100 (%)	21.1 (16.8)	21.1 (19.1)	22.3 (18.9)	23.9 (13.3)	17.1 (15.9)	0.75
COT-gluc/total COT × 100 (%)	14.8 (11.3)	10.9 (9.6)	17.0 (13.7)	12.6 (7.6)	19.5 (12.7)	0.14
3HC-gluc/total 3HC × 100 (%)	15.5 (8.3)	15.8 (6.5)	15.0 (8.9)	17.6 (10.0)	13.3 (7.9)	0.58
NIC-gluc/NIC × 100 (%)	35.0 (42.0)	36.0 (42.6)	40.9 (58.4)	35.7 (26.8)	27.3 (38.9)	0.87
COT-gluc/COT × 100 (%)	19.9 (20.6)	13.6 (13.0)	24.4 (26.7)	15.3 (10.2)	28.1 (27.0)	0.15
3HC-gluc/3HC × 100 (%)	19.5 (12.1)	19.5 (9.7)	18.8 (12.5)	23.0 (15.2)	16.2 (10.5)	0.51
3HC/COT	0.76 (0.60)	0.46 (0.26)	0.74 (0.69)	0.75 (0.30)	1.14 (0.85)[b]	0.02
[3HC + 3HC-gluc]/COT	0.90 (0.69)	0.55 (0.32)	0.87 (0.75)	0.94 (0.40)	1.33 (0.96)[b]	0.02
Percent non C-oxidation (%)	57.2 (16.2)	66.3 (11.8)	60.8 (14.1)	50.0 (14.4)[a]	50.2 (19.0)[b]	0.006
Percent C-oxidation (%)	42.8 (16.2)	33.7 (11.8)	39.2 (14.1)	50.0 (14.4)[a]	49.8 (19.0)[b]	0.006

Notes: NMR-d2: 1st quartile, 0.11; median, 0.14; 3rd quartile, 0.19

[a] $p<0.05$, 3rd quartile significantly different from 1st quartile

[b] $p<0.05$, 4th quartile significantly different from 1st quartile

[c] $p<0.05$, 4th quartile significantly different from 2nd quartile

[d] $p<0.05$, 4th quartile significantly different from 3rd quartile

Percent non C-oxidation is [NIC + NIC-gluc + NNO]/TNE × 100

Percent C-oxidation is [COT + COT-gluc + 3HC + 3HC-gluc + CNO + NNIC + NCOT]/TNE × 100

TABLE 6

Correlation between NMR and urine metabolites and metabolite ratios

	Pearson Correlation with Fisher's z transformation					
Variable	**NMR(d_0)**	**logNMR(d_0)**	**NMR(d_2)**	**logNMR(d_2)**	**NMR(d_4)**	**logNMR(d_4)**
Nicotine (NIC)	−0.01	−0.06	−0.12	−0.12	−0.14	−0.09
NIC glucuronide	−0.24	−0.43***	−0.04	−0.002	−0.23	−0.21
Cotinine (COT)	0.001	0.16	−0.01	0.05	0.14	0.20
COT glucuronide	0.24	0.28*	0.29*	0.37**	0.30*	0.36**
3-Hydroxycotinine (3HC)	0.60***	0.59***	0.67***	0.64***	0.71***	0.66***
3HC glucuronide	0.38**	0.40**	0.41**	0.41**	0.42***	0.40**
Nicotine N-oxide (NNO)	−0.60***	−0.57***	−0.55***	−0.60***	−0.59***	−0.70***
Cotinine N-oxide (CNO)	0.08	0.22	0.04	0.04	0.13	0.11
Nornicotine (NNIC)	−0.33**	−0.46***	−0.32*	−0.28*	−0.36**	−0.23
Norcotinine (NCOT)	0.11	0.20	0.08	0.09	0.13	0.13
NIC-gluc/total NIC	−0.11	−0.14	0.01	0.03	−0.07	−0.10
COTgluc/total COT	0.21	0.17	0.27*	0.35**	0.19	0.26*
3HC-gluc/total 3HC	0.01	0.06	−0.01	−0.03	0.01	0.06
NIC-gluc/NIC	−0.11	−0.13	−0.02	−0.02	−0.09	−0.16
COT-gluc/COT	0.21	0.18	0.25	0.31*	0.19	0.24
3HC-gluc/3HC	0.01	0.06	−0.01	−0.02	−0.01	0.04
3HC/COT	0.56***	0.53***	0.58***	0.54***	0.57***	0.50***
[3HC + 3HC-gluc]/COT	0.57***	0.54***	0.60***	0.55***	0.59***	0.51***
Non C-oxidation	−0.44***	−0.51***	−0.48***	−0.51***	−0.57***	−0.58***
C-oxidation	0.43***	0.51***	0.48***	0.51***	0.57***	0.58***

Notes: Non C-oxidation is [NIC + NIC-gluc + NNO]/TNE; C-oxidation is [COT + COT-gluc + 3HC + 3HC-gluc + CNO + NNIC + NCOT]/TNE;

* p<0.05;

** p<0.01;

*** p<0.001

DEPARTMENT OF THE TREASURY

ALCOHOL AND TOBACCO TAX AND TRADE BUREAU

STATISTICAL REPORT - TOBACCO

Report Date: 05-MAR-2018

Report Symbol: TTB S 5210-12-2017

Reporting Period: December 2017

(Number of Cigarettes & Cigars - Pounds of Pipe, Chewing Tobacco, Roll-Your-Own & Snuff)

Manufactured Domestically or Received from Puerto Rico	Current Month	Prior Month	Prior Year Current Month	Current Year Cumulative Year to Date	Prior Year Cumulative Year to Date
Cigarettes - Small	12,419,505,974	19,682,947,663	15,020,011,207	245,630,349,001	270,260,528,537
Cigarettes - Large	0	0	0	0	0
Cigars - Small	44,988,742	79,683,972	105,970,119	955,815,513	1,032,751,246
Cigars - Large	368,792,271	470,809,608	412,211,765	5,529,470,890	5,441,458,721
Snuff	8,688,556	9,974,421	9,056,463	120,223,566	120,498,770
Chewing Tobacco	1,156,882	7,296,237	1,207,570	23,499,403	18,954,435
Pipe Tobacco	2,517,562	2,944,372	2,622,951	36,502,414	36,824,320
Roll-Your-Own Tobacco	141,670	187,996	218,821	2,493,070	2,974,964
Removed Taxable including from Puerto Rico					
Cigarettes - Small	17,689,783,179	19,796,665,998	17,425,945,998	239,300,783,604	249,821,233,796
Cigarettes - Large	0	0	0	0	0
Cigars - Small	27,739,976	49,728,851	45,806,155	414,659,792	475,333,201
Cigars - Large <= $763.222	399,920,510	440,784,820	392,341,811	5,103,203,113	5,054,757,879
> $763.222	183,906	256,900	199,609	2,501,995	2,004,014
Total Large	400,104,416	441,041,720	392,541,420	5,105,705,108	5,056,761,893
Snuff	9,015,352	9,782,267	8,663,104	118,590,458	118,477,707
Chewing Tobacco	1,245,176	1,439,450	1,363,985	17,479,594	18,913,518
Pipe Tobacco	2,591,020	2,898,919	2,505,891	35,070,257	34,690,860
Roll-Your-Own Tobacco	143,067	217,122	255,445	2,462,689	2,938,378
Removed Tax Exempt - Cigarettes					
Small - Export	229,232,400	259,743,800	825,228,000	4,093,480,340	18,153,542,520
Transfer to Export Warehouses	99,292,000	91,343,600	89,476,800	1,097,588,600	1,230,689,600
Use of the U.S.	234,000	2,449,200	400	6,520,800	49,561,600
Personal Consumption/Experimental	2,427,029	1,360,123	1,157,010	21,228,638	21,839,221
Total Small	331,185,429	354,896,723	915,862,210	5,218,818,378	19,455,632,941
Large	0	0	0	0	0
Removed Tax Exempt - Cigars					
Small - Export	54,332,000	34,956,000	71,012,400	532,035,400	542,405,200
Transfer to Export Warehouses	0	0	0	0	0
Use of the U.S.	0	0	0	0	9,800
Personal Consumption/Experimental	8,610	14,613	6,013	173,495	186,921
Total Small	54,340,610	34,970,613	71,018,413	532,208,895	542,601,921
Large - Export	1,568,500	2,941,245	9,061,500	46,195,681	72,038,150
Transfer to Export Warehouses	55,500	38,000	0	336,500	0
Use of the U.S.	0	0	0	8,600	0
Personal Consumption/Experimental	27,854	71,060	9,906	2,453,882	310,998
Total Large	1,651,854	3,050,305	9,071,406	48,994,663	72,349,148

NOTE: Changes in figures from prior reports could be due to amended reports being filed. This data is not final and may need to be amended.

STATISTICAL REPORT - TOBACCO

TTB S 5210-12-2017

	Current Month	Prior Month	Prior Year Current Month	Current Year Cumulative Year to Date	Prior Year Cumulative Year to Date
Removed Tax Exempt - Smokeless Tobacco					
Snuff - Export & To Export Warehouses	80,016	82,695	60,509	1,157,265	1,033,794
Other	686	791	812	14,560	15,068
Chewing Tobacco - Exp. & To Exp. Whs.	0	3,086	1,620	29,127	19,883
Other	99	402	169	3,568	3,022
Removed Tax Exempt - Pipe Tobacco					
Export and To Export Warehouses	173,730	126,162	154,963	1,449,991	927,200
Other	216	354	235	2,200	2,323
Removed Tax Exempt - Roll-Your-Own Tobacco					
Export and To Export Warehouses	9,661	17,080	881	39,981	54,289
Other	7	4	5	191	163
IMPORTED FROM FOREIGN COUNTRIES Entered/Withdrawn for Consumption (As reported by the Bureau of the Census in Publication IM 146)					
Cigarettes, Total (USTSA 2402.20.1000, 2402.20.8000, 2402.20.9000)	633,143,000	557,526,000	758,366,000	7,866,487,000	7,629,000,000
Cigars - Small (USTSA 2402.10.3030, 2402.10.8030)	3,076,000	2,763,000	1,305,000	25,198,000	21,351,000
Cigars - Large: (USTSA 2402.10.3070, 2402.10.6000)	497,738,000	826,262,000	453,963,000	7,434,389,000	6,638,969,000
(USTSA 2402.10.8050, 2402.10.8080)	29,246,000	34,357,000	28,967,000	351,011,000	335,870,000
Total Large	526,984,000	860,619,000	482,930,000	7,785,400,000	6,974,839,000
Snuff (USTSA 2403.99.2040)	70,030	43,563	73,848	549,547	606,334
Chewing Tobacco (USTSA 2403.99.2030)	92,102	135,560	95,218	1,003,775	1,092,411
Pipe Tobacco (USTSA 2403.10.2020, 2403.10.2080)	507,246	48,019	432,770	3,665,466	4,899,643
Roll-Your-Own Tobacco (USTSA 2403.10.2050)	20,981	9,270	4,063	202,108	238,630
Release to Domestic Factories Without Payment of Tax (Included also in above "Entered/Withdrawn for Consumption" Category)					
Cigarettes - Small	0	0	0	28,000	816,000
Cigarettes - Large	0	0	0	0	0
Cigars - Small	0	0	0	0	0
Cigars - Large	0	0	1,500	1,116	5,220
Snuff	0	0	0	0	0
Chewing Tobacco	0	0	0	0	0
Pipe Tobacco	0	0	0	966	0
Roll-Your-Own Tobacco	0	0	0	0	80
Onhand / Close of Business					
Cigarettes - Small	21,756,253,364	27,442,937,883	21,879,112,701		
Cigarettes - Large	0	0	0		
Cigars - Small	97,552,289	134,772,133	94,824,523		
Cigars - Large	504,824,639	550,291,121	483,754,324		
Snuff	3,476,462	3,920,544	4,065,912		
Chewing Tobacco	665,213	756,850	613,098		
Pipe Tobacco	1,658,254	1,922,533	1,623,696		
Roll-Your-Own Tobacco	167,436	178,443	190,994		

NOTE: Changes in figures from prior reports could be due to amended reports being filed. This data is not final and may need to be amended.

Centers for Disease Control and Prevention
MMWR
Morbidity and Mortality Weekly Report
Weekly / Vol. 64 / No. 20
May 29, 2015

World No Tobacco Day — May 31, 2015

Each year, the tobacco epidemic kills an estimated 6 million persons worldwide, including about 600,000 who die because of secondhand smoke exposure. If current trends continue, this number is expected to reach 8 million deaths annually by 2030 (*1*).

Sponsored by the World Health Organization (WHO) and observed on May 31 each year, World No Tobacco Day highlights the health risks associated with tobacco use and encourages effective actions to reduce tobacco consumption. This year, WHO calls for international collaboration to stop the illicit trade of tobacco products (*2*).

Illicit tobacco trade is characterized by tax avoidance and tax evasion, such as bootlegging, counterfeiting, and smuggling. This practice undermines tobacco use prevention and control by increasing the accessibility and affordability of tobacco products and can reduce government tax revenue (*3*). An estimated one in 10 cigarettes consumed worldwide and 8%–21% of those consumed in the United States are illicit (*2,4*). Governments can adopt a range of measures to reduce illicit tobacco trade, as described by the WHO *Protocol to Eliminate Illicit Trade in Tobacco Products* (*3*).

References

1. Eriksen M, Mackay J, Schluger N, Gomeshtapeh F, Drope J. The tobacco atlas. Fifth ed. Brighton, UK: American Cancer Society; 2015. Available at http://www.tobaccoatlas.org.
2. World Health Organization. World No Tobacco Day: 31 May 2015. Geneva, Switzerland: World Health Organization; 2015.
3. World Health Organization. Protocol to eliminate illicit trade in tobacco products. WHO Framework Convention on Tobacco Control. Geneva, Switzerland: World Health Organization; 2013.
4. National Academy of Sciences. Understanding the U.S. illicit tobacco market: characteristics, policy context, and lessons from international experiences. Washington, DC: National Academies Press. In press 2015.

Use of Tobacco Tax Stamps to Prevent and Reduce Illicit Tobacco Trade — United States, 2014

Jamie Chriqui, PhD[1]; Hillary DeLong, JD[2]; Camille Gourdet, JD[2]; Frank Chaloupka, PhD[3]; Sarah Matthes Edwards, MSPH[4]; Xin Xu, PhD[4]; Gabbi Promoff, MA[4] (Author affiliations at end of text)

Tobacco use is the leading cause of preventable disease and death in the United States (*1*). Increasing the unit price on tobacco products is the most effective tobacco prevention and control measure (*2*). Illicit tobacco trade (illicit trade) undermines high tobacco prices by providing tobacco users with cheaper-priced alternatives (*3*). In the United States, illicit trade primarily occurs when cigarettes are bought from states, jurisdictions, and federal reservation land with lower or no excise taxes, and sold in jurisdictions with higher taxes. Applying tax stamps to tobacco products, which provides documentation that taxes have been paid, is an important tool to combat illicit trade. Comprehensive tax stamping policy, which includes using digital, encrypted ("high-tech") stamps, applying stamps to all tobacco products, and working with tribes on stamping agreements, can further prevent and reduce

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U.S. Department of Health and Human Services
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illicit trade (*4,5*). This report describes state laws governing tax stamps on cigarettes, little cigars (cigarette-sized cigars), roll-your-own tobacco (RYOT), and tribal tobacco sales across the United States as of January 1, 2014, and assesses the extent of comprehensive tobacco tax stamping in the United States. Forty-four states (including the District of Columbia [DC]) applied traditional paper ("low-tech") tax stamps to cigarettes, whereas four authorized more effective high-tech stamps. Six states explicitly required stamps on other tobacco products (i.e., tobacco products other than cigarettes), and in approximately one third of states with tribal lands, tribes required tax stamping to address illicit purchases by nonmembers. No U.S. state had a comprehensive approach to tobacco tax stamping. Enhancing tobacco tax stamping across the country might further prevent and reduce illicit trade in the United States.

The Tobacconomics Program* examined state statutes and regulations and, for tribal tobacco sales, relevant agency opinions and case law, under a cooperative agreement funded by the National Cancer Institute as part of its State and Community Tobacco Control Initiative, 2011–2015. State laws were compiled through primary legal research using the Westlaw and Lexis-Nexis commercial legal research services. Where possible, state law data were verified against publicly available secondary sources, including CDC's State Tobacco Activities Tracking and Evaluation system,† which provides current and historical state-level data on tobacco use prevention and control, including cigarette stamping. Clarification of codified law was sought through state or federal case law, Attorneys General opinions, and notices or rulings from states' departments of revenue. Excluded from the tribal sales research were state laws that made general reference to tobacco sales without explicit reference to tribes or application to tribal sales by case law, Attorneys General opinions, or departments of revenue notices; also excluded were tribal codes, tax agreements, or compacts not codified by the state (i.e., individual tribe-specific codes and policies).

As of January 1, 2014, a total of 48 states (including DC) applied cigarette tax stamps. Only four of these authorized the use of high-tech stamps. Three of these four states (California, Massachusetts, and Michigan) have implemented their use; New Jersey has not (Table). Of the 17 states that taxed little cigars at an amount equivalent to cigarettes, which makes them subject to stamping, only five of these states' laws explicitly required stamps on little cigars. Of the five states that taxed RYOT as cigarettes, which makes them subject to stamping, only two explicitly required stamps on RYOT (Table, Figure 1).

Although Native American tribes within the United States are protected by sovereign immunity and states do not have legal authority over tribes within their borders, agreements,

* Tobacconomics Program, Health Policy Center, Institute for Health Research and Policy, University of Illinois at Chicago. Additional information available at http://www.tobacconomics.org.

† Information available at http://www.cdc.gov/tobacco/data_statistics/state_data/state_system/index.htm.

The *MMWR* series of publications is published by the Center for Surveillance, Epidemiology, and Laboratory Services, Centers for Disease Control and Prevention (CDC), U.S. Department of Health and Human Services, Atlanta, GA 30329-4027.

Suggested citation: [Author names; first three, then et al., if more than six.] [Report title]. MMWR Morb Mortal Wkly Rep 2015;64:[inclusive page numbers].

TABLE. States with laws requiring tax stamps on cigarettes, little cigars (LC), roll-your-own tobacco (RYOT), and tribal tobacco — United States, January 1, 2014

State (and District of Columbia)	Cigarettes		LC and RYOT		Tribal stamping	
	Stamp required	Encrypted tax stamp	LC and/or RYOT taxed as a cigarette*	LC and/or RYOT explicitly stamped	On-reservation tobacco sales require stamps on some or all products	Type of stamp(s) required
Alabama	Yes					
Alaska	Yes				—†	
Arizona	Yes				Yes (all§)	SE, GT, O¶
Arkansas	Yes		RYOT			
California	Yes	Yes	LC			
Colorado	Yes					
Connecticut	Yes					
Delaware	Yes					
District of Columbia	Yes		LC			
Florida	Yes				Yes (all§)	Silent**
Georgia	Yes					
Hawaii	Yes		LC			
Idaho	Yes				Yes (some††)	SE
Illinois	Yes		LC	LC		
Indiana	Yes					
Iowa	Yes		LC	LC	Prohibited§§	
Kansas	Yes					
Kentucky	Yes					
Louisiana	Yes					
Maine	Yes					
Maryland	Yes					
Massachusetts	Yes	Yes	LC	LC		
Michigan	Yes	Yes			—†	
Minnesota	Yes		LC		Yes (some††)¶¶***	SE, TA
Mississippi	Yes					
Missouri	Yes					
Montana	Yes		LC		Yes (some††)***	SE
Nebraska	Yes				Yes (all§)	SE, ST
Nevada	Yes				Yes (all§)	SE, GT
New Hampshire	Yes		LC, RYOT			
New Jersey	Yes	Yes†††				
New Mexico	Yes		LC, RYOT		Yes (all§)	SE, GT§§§
New York	Yes		LC		Yes (all§)	SE
North Carolina	No					
North Dakota	No				—†	

See table footnotes on page 544.

such as ones to regulate tobacco sales, may be negotiated. Thirty-four states have federal reservation land within their borders. Of these, 20 regulated tribal tobacco sales as of January 1, 2014, 13 of which explicitly addressed stamping of products sold on-reservation (Table, Figure 2). Of those 13, nine required stamps on all cigarettes or tobacco products sold on-reservation, and four only required stamps on products sold to nonmembers of the tribe or on all products sold by tribes without tax agreements with the state.

Discussion

This report indicates that although the majority of states required low-tech cigarette tax stamps as of January 1, 2014, few were using high-tech stamps, applying stamps to other tobacco products, or working with tribes on stamping agreements. Depending on analytical approaches and definitions of illicit trade, it is estimated that 8%–21% of cigarettes consumed in the United States are purchased illicitly (*4*). These illicit purchases undermine tobacco control efforts (*2*), might contribute to health disparities (*4*), and reduce local and state revenues by billions of dollars annually (*4*). Lack of comprehensive tax stamping could thwart U.S. efforts to reduce illicit trade and complicate law enforcement.

Three states (North Carolina, North Dakota, and South Carolina) did not require any stamps, making tax collection more difficult and potentially facilitating illicit trade. The majority of states use low-tech stamps on cigarettes, which are easier to counterfeit (*6*). These conventional stamps do not take advantage of overt and covert security features and encrypted information regarding manufacturing, distribution, and retail destination (*4*) that is contained in high-tech stamps. A recent study of littered cigarette packs in New York City found that approximately 60% of packs examined lacked the appropriate tax stamp (*7*), which was more prevalent in

TABLE. (*Continued*) States with laws requiring tax stamps on cigarettes, little cigars (LC), roll-your-own tobacco (RYOT), and tribal tobacco — United States, January 1, 2014

State (and District of Columbia)	Cigarettes		LC and RYOT		Tribal stamping	
	Stamp required	Encrypted tax stamp	LC and/or RYOT taxed as a cigarette*	LC and/or RYOT explicitly stamped	On-reservation tobacco sales require stamps on some or all products	Type of stamp(s) required
Ohio	Yes					
Oklahoma	Yes				Yes (all§)	SE, GT, TA
Oregon	Yes				—†	
Pennsylvania	Yes		LC			
Rhode Island	Yes		LC	LC		
South Carolina	No		LC			
South Dakota	Yes				—†	
Tennessee	Yes					
Texas	Yes					
Utah	Yes		LC		Yes (some††)¶¶	SE
Vermont	Yes		LC, RYOT	LC, RYOT		
Virginia	Yes					
Washington	Yes		LC, RYOT	RYOT	Yes (all§)	SE, ST, TA
West Virginia	Yes					
Wisconsin	Yes				Yes (all§)	SE, GT
Wyoming	Yes				Prohibited§§	
Totals	**48**	**4**	**18**	**6**	**13**	—

Source: Tobacconomics Program, Health Policy Center, Institute for Health Research and Policy, University of Illinois at Chicago. Additional information available at http://www.tobacconomics.org.

Abbreviations: SE = state excise stamp; GT = general tribal stamp (used by all tribes); O = other; TA = tribal agreement stamp (used by all tribes with tribal agreement); ST = specific tribal stamp (specific to certain tribe).

* In these states, LC and/or RYOT are taxed as cigarettes and, therefore, with the exception of LC in South Carolina (where cigarettes are not stamped), might be subject to cigarette stamping requirements.

† State regulates tribal tobacco sales but is silent on the stamping issue.

§ State laws explicitly state that all cigarettes or tobacco products sold on-reservation require stamps.

¶ Tax-free reservation stamp.

** Law is silent on specific stamps required for tribal sales.

†† In certain instances (e.g., products sold to nonmembers or products sold to tribes without tax agreements), cigarettes or tobacco products sold on-reservation require stamps.

§§ Stamps explicitly prohibited on cigarettes or tobacco products sold on-reservation.

¶¶ Tax stamps required on products sold to nonmembers.

*** Tax stamps required on products sold to tribes without agreements.

††† Authorized by law but not currently implemented.

§§§ New Mexico has a general tribal tax-exempt stamp (for tribal members) and a tax credit stamp (for sales to nonmembers on reservation).

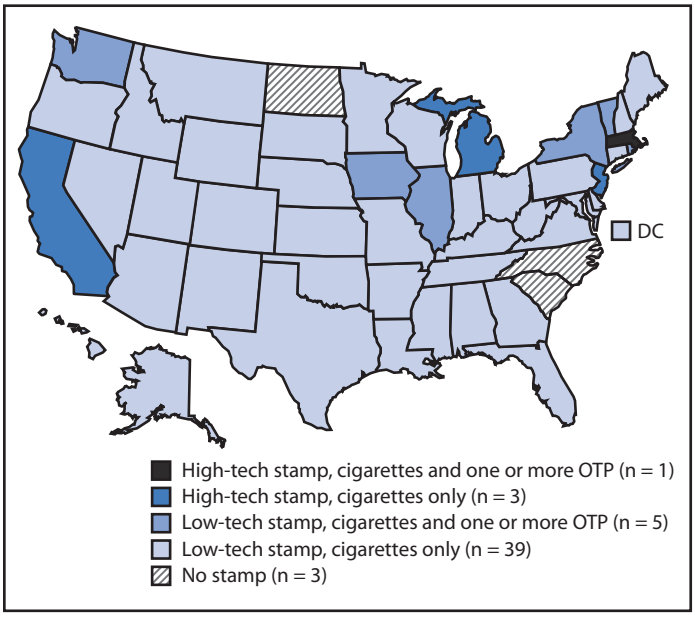
socioeconomically deprived areas, suggesting that illicit trade might exacerbate existing health disparities by facilitating access to cigarettes and making them more affordable to persons with lower incomes (*7*).

A few states are successfully employing high-tech stamps (*4*). Anti-counterfeit technology enables enforcement agents to immediately authenticate the stamp and to detect counterfeit stamps. A study in California showed that the additional tax revenues collected using the state's high-tech stamp could be as much as eight times higher than implementation and administrative costs (*4*).

Although most states applied at least low-tech stamps to cigarettes, only a few expressly stamped little cigars or RYOT. Requiring stamps on other tobacco products, especially cigarette analogues such as little cigars and RYOT, is an important aspect of preventing tax avoidance by minimizing opportunities and incentives for substitution (*2*). Without stamps, it is difficult for inspectors to distinguish tobacco products on which tax has been paid from those coming from illicit markets.

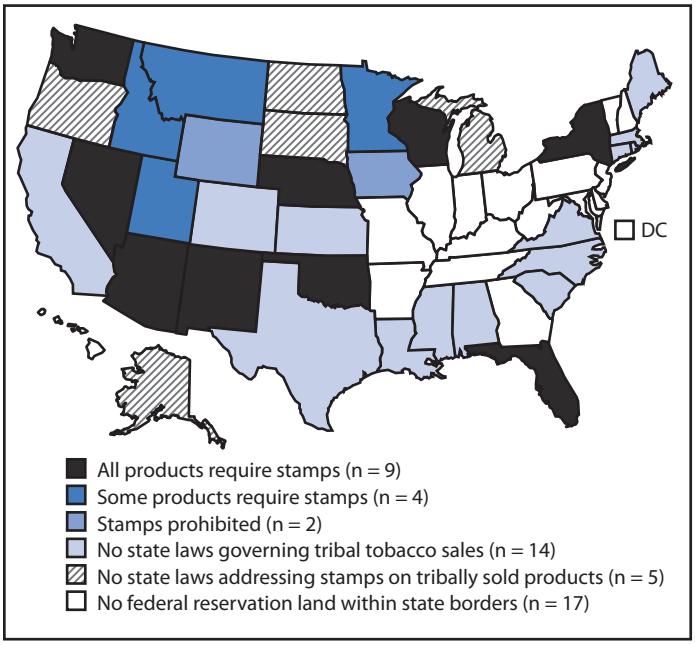
A critical facet of a comprehensive approach to tobacco stamping is the inclusion of all sources of tobacco in this practice, including sales by Native American tribes. Several states have entered into agreements with Native American tribes on general tobacco-related issues or have negotiated specific tax agreements with tribes to reduce the avoidance of tobacco excise taxes by nonmembers, including application of tax stamps to products sold on-reservation. Although tribal members who purchase tobacco on-reservation are exempt from state taxation, nonmembers purchasing on-reservation are not exempt from state taxation; these illegal purchases by nonmembers are a significant source of illicit trade because of challenges in collecting taxes on sales to nonmembers (*8*). Agreements requiring stamp application or a state's decision to apply stamps strategically within the distribution chain might alleviate concerns about tax losses from tribal sales, because it

FIGURE 1. Use and type of cigarette and other tobacco product (OTP) stamps, by state — United States, January 1, 2014



Source: Tobacconomics Program, Health Policy Center, Institute for Health Research and Policy, University of Illinois at Chicago. Additional information available at http://www.tobacconomics.org.

FIGURE 2. Laws governing use of tobacco stamps on tobacco products sold on tribal reservations, by state — United States, January 1, 2014



Source: Tobacconomics Program, Health Policy Center, Institute for Health Research and Policy, University of Illinois at Chicago. Additional information available at http://www.tobacconomics.org.

What is already known on this topic?

Increasing the unit price on tobacco products is the most effective tobacco prevention and control intervention, especially among price-sensitive populations, such as youth. Illicit tobacco trade can undermine the effectiveness of high tobacco prices by providing tobacco users with cheaper priced alternatives. Tobacco tax stamping is intended to further support efforts to prevent and reduce illicit trade.

What is added by this report?

A comprehensive tax stamping approach includes the use of digital, encrypted ("high-tech") stamps, the application of stamps to all tobacco products, including little cigars and roll-your-own tobacco; and working with Native American tribes on stamping agreements. As of January 1, 2014, most states used traditional paper ("low-tech") stamps that are easy to counterfeit, and many did not explicitly require stamps on cigarette-equivalent products such as little cigars and roll-your-own tobacco. Approximately two thirds of states with federal reservation land did not have codified agreements that permit tobacco stamping of tribally sold products.

What are the implications for public health practice?

Illicit trade undermines tobacco control efforts and might contribute to health disparities. Comprehensive tax stamping policies could enhance U.S. efforts to reduce illicit trade, thereby increasing revenues as well as protecting public health and reducing smoking by stopping illegal cigarette sales.

encourages prepayment of taxes, and might aid in enforcement of excise tax payment by establishing clear procedures and tax rates for products sold on federal reservation land.

The findings in this report are subject to at least three limitations. First, the cigarette, little cigar, and RYOT data were limited to codified statutory and administrative law and do not include Attorneys General opinions, case law, or departments of revenue–issued notices, rulings, or decisions. For example, California's statutes or regulations do not explicitly call for little cigar stamping. However, per a notice issued by California's Board of Equalization (excluded from this report's primary legal research), all little cigars must be stamped.[§] Second, this report did not include information on states that maintain general tobacco sales laws that are not explicitly enforced with tribal entities, and it was not possible to determine whether the states that regulate tribal tobacco sales, but do not explicitly address stamping do, in fact, include stamps in their noncodified agreements or compacts. In addition, a tribe's own laws might dictate tribal tax rates or enforcement mechanisms not captured in this report. Finally, this report only reviewed the laws pertaining to the use of tax stamps on tobacco products; however, tax stamping on its own is not sufficient to deter illicit

[§] Information available at http://www.boe.ca.gov.

trade. Enforcement is also necessary (*5,6*). Other policy interventions, such as licensing, implementing a track-and-trace system, and the harmonization of tax codes, also contribute to reductions in illicit trade (*3*).

A comprehensive approach to tobacco tax stamping could be an important tool in reducing illicit trade and revenue loss in the United States. Applying tax stamps to all tobacco products, and for those states with federal reservation land within their borders, working with tribes to negotiate mutually beneficial agreements, including the use of stamps on tobacco products sold on reservation land, could have an important impact on reducing illicit trade and further reduce smoking and associated health care costs as well as recoup lost revenues from illicit trade (*4*). Additionally, introducing high-tech tax stamps with new technologies including encryption, holograms, and scannable barcodes in all states could further reduce counterfeiting and improve supply-chain monitoring and enforcement (*4*).

Acknowledgment

National Cancer Institute, National Institutes of Health (grant no. U01CA154248).

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References

1. US Department of Health and Human Services. The health consequences of smoking—50 years of progress: a report of the Surgeon General. Atlanta, GA: US Department of Health and Human Services, CDC; 2014. Available at http://www.surgeongeneral.gov/library/reports/50-years-of-progress/full-report.pdf.
2. Chaloupka FJ, Yurekli A, Fong GT. Reviews: tobacco taxes as a tobacco control strategy. Tob Control 2012;21:172–80.
3. Joossens L, Raw M. From cigarette smuggling to illicit tobacco trade. Tob Control 2012;21:230–4.
4. National Academy of Sciences. Understanding the U.S. illicit tobacco market: characteristics, policy context, and lessons from international experiences. Washington, DC: National Academies Press. In press 2015.
5. World Health Organization. Framework Convention on Tobacco Control: protocol to eliminate illicit trade in tobacco products. Geneva, Switzerland: World Health Organization; 2013. Available at http://apps.who.int/iris/bitstream/10665/80873/1/9789241505246_eng.pdf.
6. Allen E. The illicit trade in tobacco products and how to tackle it. World Customs Journal 2012;6:121–9.
7. Kurti MK, Von Lampe K, Thomkins DE. The illegal cigarette market in a socioeconomically deprived inner-city area: the case of the South Bronx. Tob Control 2013;22:138–40.
8. Alderman J. Strategies to combat illicit trade. Available at http://www.publichealthlawcenter.org/sites/default/files/resources/tclc-syn-smuggling-2012_0.pdf.

Approaches for Controlling Illicit Tobacco Trade — Nine Countries and the European Union

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An estimated 11.6% of the world cigarette market is illicit, representing more than 650 billion cigarettes a year and $40.5 billion in lost revenue (*1*). Illicit tobacco trade refers to any practice related to distributing, selling, or buying tobacco products that is prohibited by law, including tax evasion (sale of tobacco products without payment of applicable taxes), counterfeiting, disguising the origin of products, and smuggling (*2*). Illicit trade undermines tobacco prevention and control initiatives by increasing the accessibility and affordability of tobacco products, and reduces government tax revenue streams (*2*). The World Health Organization (WHO) *Protocol to Eliminate Illicit Trade in Tobacco Products*, signed by 54 countries, provides tools for addressing illicit trade through a package of regulatory and governing principles (*2*). As of May 2015, only eight countries had ratified or acceded to the illicit trade protocol, with an additional 32 needed for it to become international law (i.e., legally binding) (*3*). Data from multiple international sources were analyzed to evaluate the 10 most commonly used approaches for addressing illicit trade and to summarize differences in implementation across select countries and the European Union (EU). Although the WHO illicit trade protocol defines shared global standards for addressing illicit trade, countries are guided by their own legal and enforcement frameworks, leading to a diversity of approaches employed across countries. Continued adoption of the methods outlined in the WHO illicit trade protocol might improve the global capacity to reduce illicit trade in tobacco products.

Data on approaches for addressing illicit trade were obtained from a combination of sources from individual countries, including literature searches, reports by international agencies and nongovernmental organizations, industry documents, online data sources by agencies that oversee enforcement, and interviews with in-country experts.* The following 10 most commonly identified approaches were evaluated: 1) licensing, 2) product markers, 3) national recordkeeping, 4) track-and-trace systems, 5) enforcement, 6) export tax, 7) tax harmonization, 8) agreements with tobacco industry, 9) promotion of public awareness, and 10) coordination among agencies. The status of these approaches was assessed in nine countries (Brazil, Canada, Hungary, Italy, Malaysia, Romania, Spain, Turkey, and the United Kingdom [UK]), and EU. These countries were selected based on data availability and participation in the WHO Framework Convention on Tobacco Control (FCTC). EU is described separately from its member states because current approaches used by individual member states may differ from the central EU action plan. Approaches were assessed as of January 2015.

The most common anti-illicit–trade measures were licensing and enforcement (Table 1), which were present in all countries reviewed in this report (Table 2). A total of nine countries employed product markers, most commonly in the form of tax stamps (Table 2). Although requirements for product markers are not included in the centralized EU Tobacco Products Directive, EU member states have incorporated those on an individual basis. Systems for national recordkeeping and agency coordination were established in all countries except Malaysia. Track-and-trace systems, as outlined in the WHO illicit trade protocol, were in effect in Brazil and Turkey, and, in a limited version, in Canada and Hungary; EU and its member states operate a separate system for monitoring the movement of excise goods across their borders. Tax harmonization was employed within EU. Agreements with the tobacco industry were in place in most countries, except for Brazil and Malaysia. Public awareness programs were not widely employed, and export taxes were applied in Brazil and Canada only. While all examined countries were parties to the WHO FCTC, most have not yet ratified or acceded to (i.e., made legally binding) the WHO illicit trade protocol, and only one has thus far acquired accession status (Table 2) (*3*).

Discussion

Approaches to address illicit tobacco trade vary across countries. In the sample of countries in this report, the most commonly used approaches included licensing, markers, national recordkeeping, and enforcement, while other measures such as track-and-trace systems and export taxes were not universally employed. Research suggests that the revenue gains from eliminating illicit tobacco trade globally would exceed $31 billion, and might help prevent more than 160,000 tobacco-related deaths per year from 2030 onwards (*1*). Accordingly, continued adoption of the provisions outlined in the WHO illicit trade protocol

*Additional information on sources by country is available at http://tobacconomics.org.

TABLE 1. Definitions of common approaches to address illicit tobacco trade

Approach	Definition
Licensing	Official authorization for engaging in any activity within the tobacco supply chain, from tobacco growing to product manufacturing to product transportation, retail, and export
Markers	Counterfeit-resistant, affixed images on product packaging, most commonly in the form of tax stamps, which indicate date and location of manufacture and the intended retail market
National recordkeeping	Collection of data on the tax liability of tobacco products within country borders or while transiting through individual countries
Track-and-trace	Systems incorporating both markers and national recordkeeping structures to enable tracking of tobacco products throughout the supply chain; tracing the movement of products by transferring tracking data into a global information-sharing database
Enforcement	Commitment to detect and prosecute illicit trade activity
Export tax	Applying a cigarette export tax to reduce the motivation for illegal re-import of exported products
Tax harmonization	Equalizing tax rates across neighboring jurisdictions to lower cigarette price differences across borders
Agreements with industry	Obtaining industry cooperation in improving the security of the supply chain
Public awareness	Disseminating information about the risks associated with illicit tobacco trade; motivating support for enforcement activities
Agency coordination	Coordination between agencies within and across borders to support intelligence gathering, joint customs operations, and sharing of best practices

TABLE 2. Implementation of common approaches to address illicit tobacco trade and year of ratification of WHO Framework Convention for Tobacco Control (FCTC) and signing/accession of WHO FCTC *Protocol to Eliminate Illicit Trade in Tobacco Products*, by nine countries and the European Union (EU)

Approach	Brazil	Canada	EU	Hungary	Italy	Malaysia	Romania	Spain	Turkey	UK
Licensing	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Markers	yes	yes		yes	yes	yes	yes	yes	yes	yes
National recordkeeping	yes	yes	yes	yes	yes		yes	yes	yes	yes
Track-and-trace	yes	yes		yes					yes	
Enforcement	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Export tax	yes	yes								
Tax harmonization			yes	yes	yes		yes	yes		yes
Agreements with industry		yes	yes	yes	yes		yes	yes	yes	yes
Public awareness		yes	yes			yes				yes
Agency coordination	yes	yes	yes	yes	yes		yes	yes	yes	yes
Year ratified WHO FCTC	2005	2004	2005	2004	2008	2005	2006	2005	2004	2004
Year signed/year of accession* WHO illicit trade protocol			2013					2013/2014	2013	2013

Abbreviations: UK = United Kingdom; WHO FCTC = World Health Organization Framework Convention for Tobacco Control.
* Accession is an act by which a state signifies its agreement to be legally bound by the terms of a particular treaty.

and its accession could improve the global capacity to reduce illicit trade in tobacco products and enhance public health.

The WHO illicit trade protocol contains three main elements for addressing illicit trade: 1) controlling the supply chain of tobacco products through track-and-trace systems (Articles 6–13); 2) addressing unlawful conduct and criminal offenses through enforcement means such as seizure and disposal of confiscated products (Articles 14–19); and 3) promoting international cooperation through information sharing, mutual administrative and legal assistance, and extradition (Articles 20–31) (*2*). The WHO illicit trade protocol emphasizes the importance of national track-and-trace systems, and recommends collection of data on supply-chain movements into a global information sharing database, which would facilitate the coordination of international response (*4*). Although establishing track-and-trace systems has been identified as a central approach for limiting illicit trade, its implementation is not yet widespread. Some countries may not have the resources to support a fully functioning track-and-trace system, or they may have alternative structures already in place. For example, EU has implemented a substitute computerized system, the Excise Movement and Control System, which differs from the standard track-and-trace model by collecting only limited information in excisable goods, not monitoring duty-paid products, and relaxing the requirement for product markers. Some countries and EU employ agreements with

tobacco companies to limit tax evasion, but evidence suggests that the industry-operated monitoring system is subject to limited transparency and insufficient tracing capabilities (*5*). Turkey is among the countries that have recently implemented track-and-trace systems with noted success; the size of the illicit market has been controlled despite ongoing increases in tobacco taxes in the country (*6*,*7*).

The context for illicit tobacco trade globally varies by country. For example, while cross-border smuggling is a primary concern for many countries, the U.S. tobacco market is primarily affected by illicit domestic movement of goods from low-tax to high-tax jurisdictions (*8*). International experience with tax harmonization across jurisdictions, such as that employed in EU, can provide an example of potential strategies for reducing the size of the domestic illicit market in the United States. Because higher cigarette prices are a primary method for reducing tobacco use (*9*), an effort to reconcile tax differences across jurisdictions at a shared higher level might help limit tobacco use as well as illicit trade incentives in the United States and other countries.

This report is subject to several limitations. First, it provides a brief summary from a limited number of countries; thus, experiences and approaches from other countries might vary. Second, only the reported presence or absence of an approach was assessed, and differences across countries in the strength of implementation or enforcement were not identified.

Tobacco use is the leading preventable cause of death and disability around the globe, contributing to six million deaths per year (*10*). Illicit trade in tobacco products undermines global tobacco prevention and control interventions. This report illustrates the diversity of approaches for limiting illicit tobacco trade in a number of countries and EU. These findings underscore the importance of continued adoption of the provisions outlined in the WHO illicit trade protocol to improve the global capacity to reduce illicit trade in tobacco products. Once legally binding (ratified by at least 40 countries), the WHO illicit trade protocol will facilitate international cooperation, a core provision to counteract illicit trade. Further, continued monitoring of the implementation of the WHO illicit trade protocol could counteract the negative economic, societal, and health effects of illicit tobacco trade. Understanding differences across countries in the implementation of the WHO FCTC *Protocol to Eliminate Illicit Trade in Tobacco Products* is important for assessing country-specific needs in implementing this protocol and for identifying best practices that address illicit tobacco trade and reduce tobacco-related disease and death globally.

What is already known on this topic?

Illicit trade in tobacco undermines tobacco control efforts. The WHO Framework Convention on Tobacco Control (FCTC) *Protocol to Eliminate Illicit Trade in Tobacco Products* provides tools for addressing illicit tobacco trade through a package of regulatory and governing principles, and requires FCTC signatories to institute global track-and-trace systems and a global information sharing focal point.

What is added by this report?

There is diversity in the adoption of anti-illicit-trade measures by countries, demonstrating cross-country similarities and differences in main approaches to the standards outlined in the WHO FCTC *Protocol to Eliminate Illicit Trade in Tobacco Products*.

What are the implications for public health practice?

Continued adoption of the methods outlined in the WHO *Protocol to Eliminate Illicit Trade in Tobacco Products* can improve the global capacity to reduce illicit trade in tobacco products and enhance public health. Understanding differences across countries in the status of implementation of the WHO protocol is important for assessing country-specific needs in implementing it, and for identifying best practices in addressing illicit trade.

Acknowledgments

Rebecca Bunnel, PhD, Shanna Cox, MSPH, Brian King, PhD; Timothy McAffee, MD, Office on Smoking and Health, National Center for Chronic Disease Prevention and Health Promotion, CDC; Vera Costa E Silva, MD, PhD, World Health Organization.

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References

1. Joossens L, Merriman D, Ross H, Raw M. The impact of eliminating the global illicit cigarette trade on health and revenue. Addiction; 2010. Available at http://onlinelibrary.wiley.com/doi/10.1111/j.1360-0443.2010.03018.x/epdf.
2. World Health Organization. Protocol to eliminate illicit trade in tobacco products. Geneva, Switzerland: FCTC — WHO Framework Convention on Tobacco Control; 2013. Available at http://www.who.int/fctc/protocol/en/.
3. United Nations. Protocol to eliminate illicit trade in tobacco products, Seoul, 12 November 2012. New York, NY: United Nations Treaty Collection; 2015. Available at https://treaties.un.org/pages/ViewDetails.aspx?src=TREATY&mtdsg_no=IX-4-a&chapter=9&lang=en.
4. World Health Organization. Combating the illicit trade in tobacco products from a European perspective. Geneva, Switzerland: FCTC – WHO Framework Convention on Tobacco Control; 2014. Regional Studies Series Paper R/3. Available at http://www.who.int/fctc/publications/Regional_studies_paper_3_illicit_trade.pdf?ua=1.

5. Joossens L, Gilmore AB. The transnational tobacco companies' strategy to promote Codentify, their inadequate tracking and tracing standard. Tob Control; 2013. Available at http://tobaccocontrol.bmj.com/content/early/2013/04/26/tobaccocontrol-2012-050796.full.
6. Tayyan U. Tobacco Banderol System application in Turkey. In: Presentation Data from the 10th Asian Pacific Conference on Tobacco or Health, August 18–21, 2013; Chiba, Japan. Available at http://www.apact.jp/presentation_data/pdf/PL3-4.pdf.
7. Euromonitor International. Passport tobacco. London: Euromonitor International; 2015.
8. Ross H. Controlling illicit tobacco trade: international experience. Chicago, IL: University of Illinois at Chicago, Health Policy Center, Institute for Health Research and Policy. Tobacconomics. In press 2015.
9. International Agency for Research on Cancer. Effectiveness of tax and price policies in tobacco control Lyon, France: World Health Organization, International Agency for Research on Cancer; 2011. IARC handbooks of cancer prevention: tobacco control, Vol. 14. Available at http://www.iarc.fr/en/publications/pdfs-online/prev/handbook14/handbook14.pdf.
10. US Department of Health and Human Services. The health consequences of smoking—50 years of progress: a report of the surgeon general. Atlanta, GA: US Department of Health and Human Services, CDC; 2014. Available at http://www.surgeongeneral.gov/library/reports/50-years-of-progress/full-report.pdf.

Occupational Fatalities During the Oil and Gas Boom — United States, 2003–2013

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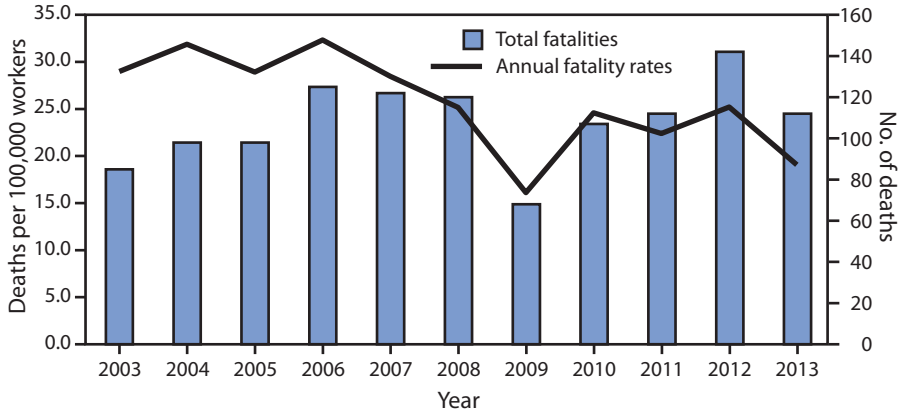
During 2003–2013, the U.S. oil and gas extraction industry experienced unprecedented growth, doubling the size of its workforce and increasing the number of drilling rigs by 71% (*1*,*2*). To describe fatal events among oil and gas workers during this period, CDC analyzed data from the Bureau of Labor Statistics (BLS) Census of Fatal Occupational Injuries (CFOI), a comprehensive database of fatal work injuries (*3*). During 2003–2013, the number of work-related fatalities in the oil and gas extraction industry increased 27.6%, with a total of 1,189 deaths; however, the annual occupational fatality rate significantly decreased 36.3% ($p<0.05$) during this 11-year period. Two-thirds of all worker fatalities were attributed to transportation incidents (479, [40.3%]) and contact with objects/equipment (308 [25.9%]). More than 50% of persons fatally injured were employed by companies that service wells (615 [51.7%]). It is important for employers to consider measures such as land transportation safety policies and engineering controls (e.g., automated technologies) that would address these leading causes of death and reduce workers' exposure to hazards (*4*–*6*).

Publicly available data from CFOI were used to determine the number of fatal injuries to workers in the U.S. land-based and offshore oil and gas extraction industry during 2003–2013. CFOI collects information from multiple data sources to identify, verify, and describe fatal work injuries (*3*). According to CFOI, a fatal injury is considered work-related if 1) the event leading to the injury occurred while the employee was working and 2) the event is verified by at least two independent data sources.* The North American Industry Classification System (NAICS) was used to identify fatal events among the three types of companies in the oil and gas extraction industry: oil and gas operators that control and manage leased areas (NAICS 211), drilling contractors that drill the wells (NAICS 213111), and well-servicing companies that provide all other types of support operations that prepare a well for production and completion (NAICS 213112).

Annual occupational fatal injury rates were calculated using worker estimates from the BLS Quarterly Census of Employment and Wages (*1*). Annual and overall fatality rates were also calculated by event type according to the Occupational Injury and Illness Classification System and by company type using NAICS. Negative binomial regression was used to estimate rates. The percent rate of change, incident rate ratio, and corresponding confidence intervals were calculated for the 11-year period, the five most frequent fatal events, and by company type. Each company and event type represents separate regression models.

During 2003–2013, 1,189 oil and gas extraction industry employees died while working, resulting in an average of 108 deaths per year and an annual average occupational fatality rate of 25.0 deaths per 100,000 workers. The highest fatality rate occurred in 2006 (32.4 deaths per 100,000 workers) with 125 fatalities (Figure). During this period all but 10 decedents were male, and the largest numbers of deaths were to workers aged 25–34 years (331 [27.8%]). Most were non-Hispanic whites (844 [71.0%]). Two-thirds of the fatalities were attributed to transportation incidents (479 [40.3%]) and contact with objects/equipment (308 [25.9%]). Incidents on land (as opposed to air or water) made up 86.2% of the transportation events. The remainder of the most frequent events were the result of fires or explosions (170, [14.3%]); exposure to harmful

FIGURE. Number* and rate† of fatal injuries among workers in the oil and gas extraction industry, by year — United States, 2003–2013§



Sources: U.S. Department of Labor, Bureau of Labor Statistics, Census of Fatal Occupational Injuries (2003–2013) and U.S. Department of Labor, Bureau of Labor Statistics, Quarterly Census of Employment and Wages (2003–2013).

* N = 1,189.

† Fatality rate calculated per 100,000 workers; significant decrease in fatality rate during 2003–2013 (negative binomial regression chi-square = 0.057; $p<0.01$).

§ 2013 data are preliminary.

* Additional information is available at http://www.bls.gov/iif/oshcfdef.htm.

substances or environments (105 [8.8%]); or falls, slips, and trips (97 [8.2%]). The largest number of fatalities occurred among workers employed by well-servicing companies (615), followed by drilling contractors (378), and operators (196); but the highest fatality rate was among workers employed by drilling companies (44.6 per 100,000 workers), followed by well-servicing companies (27.9), and operators (11.6) (Table 1).

Although the oil and gas extraction industry's number of occupational fatalities increased 27.6% during the 11-year period, it did not increase as much as the number of workers, resulting in a significant decrease in the fatality rate of 36.3% (Table 2). The average annual decrease was 4% per year (Table 1). Oil and gas operators experienced the largest decrease in the rate of fatal injuries, 8% per year (p<0.01), followed by well-servicing companies (4% per year, p<0.05). Among event types, contact with objects/equipment experienced the greatest decrease, 9% per year (p<0.001); transportation events also showed a significant decrease, 3% per year (p<0.05).

Discussion

Previous research found a positive correlation between the level of activity (number of active drilling rigs) and the occupational fatality rate in the U.S. oil and gas extraction industry (*7*). This report found that although the number of

TABLE 1. Trends* in worker fatality rates in the oil and gas extraction industry, by company type and event type, using an unadjusted model — United States, 2003–2013†

Company/event type	No.	(%)	Fatality rate§	% Rate change¶	IRR	(95% CI)	p-value
Total Fatalities	**1,189**		**25.0**	**-36.3**	**0.956**	**(0.932–0.980)**	**0.000****
By company (NAICS code)							
Operators (211)	196	(16.5)	11.6	-58.2	0.917	(0.869–0.967)	0.001**
Drilling contractors (213111)	378	(31.8)	44.6	-27.2	0.969	(0.931–1.008)	0.118
Well-servicing companies (213112)	615	(51.7)	27.9	-33.4	0.960	(0.962–0.996)	0.028**
By event††							
Transportation	479	(40.3)	10.1	-28.1	0.968	(0.938–0.998)	0.040**
Contact with objects/equipment	308	(25.9)	6.5	-60.8	0.910	(0.879–0.944)	0.000**
Fires/explosions§§	170	(14.3)	3.6	-41.3	0.948	(0.884–1.017)	0.137
Exposure to harmful environments/substances§§	104	(8.7)	2.2	-42.6	0.946	(0.890–1.006)	0.076
Falls§§	97	(8.2)	2.0	+26.8	1.024	(0.960–1.093)	0.469

Sources: U.S. Department of Labor, Bureau of Labor Statistics, Census of Fatal Occupational Injuries (2003–2013) and U.S. Department of Labor, Bureau of Labor Statistics, Quarterly Census of Employment and Wages (2003–2013).
Abbreviations: CI = confidence interval; IRR = incident rate ratio; NAICS = North American Industry Classification System.
* Determined by negative binomial regression analyses.
† Data for 2013 are preliminary.
§ Annual average fatality rate per 100,000 workers.
¶ Using predicted values from negative binomial regressions over 11 years.
** Statistically significant at p<0.05.
†† Break in Occupational Injury and Illness Classification System series in 2011.
§§ Contain one or more years during which the number of fatalities was <10.

TABLE 2. Annual fatality rates among workers in the oil and gas extraction industry, by company type and event type — United States, 2003–2013*†

	Fatality rates (yr)											
Company/event type	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	% Rate change§
Total	**29.0**	**31.9**	**29.0**	**32.4**	**28.5**	**25.2**	**16.1**	**24.6**	**22.4**	**24.5**	**19.1**	**-36.3**
By company												
Operators	14.1	23.9	13.5	16.3	10.3	13.1	7.5	7.6	7.6	13.8	6.1	-58.2
Drilling contractors	50.5	52.3	51.0	45.1	49.7	32.4	42.8	63.1	47.0	42.2	25.7	-27.2
Well-servicing companies	34.7	30.4	32.3	39.2	33.0	30.9	14.0	23.8	24.0	27.3	25.6	-33.4
By event												
Transportation	10.6	14.7	10.1	11.2	11.7	10.3	6.4	9.4	10.2	11.0	7.5	-28.1
Contact with objects/equipment	8.9	9.5	8.0	10.1	9.4	6.3	5.0	4.8	5.2	4.3	4.3	-60.8
Fires/explosions	6.5	3.6	3.6	5.4	2.3	3.8	1.4¶	5.8	2.4	4.1	2.2	-41.3
Exposure to harmful environments/ substances	1.7¶	1.6¶	3.3	3.1	3.5	2.5	2.1¶	2.3	1.8¶	1.4¶	1.4¶	-42.6
Falls	1.4¶	2.6¶	3.0	2.1¶	1.4¶	1.9¶	0.7¶	1.6¶	2.0	3.2	2.4	+26.8

Sources: U.S. Department of Labor, Bureau of Labor Statistics, Census of Fatal Occupational Injuries (2003–2013). U.S. Department of Labor, Bureau of Labor Statistics, Quarterly Census of Employment and Wages (2003–2013).
* 2013 data are preliminary.
† Break in Occupational Injury and Illness Classification System series in 2011.
§ Using predicted values from negative binomial regressions over 11 years.
¶ Contain one or more years during which the number of fatalities was <10.

active drilling rigs increased by 71% and the number of oil and gas extraction workers more than doubled (*1*,*2*) during 2003–2013, the industry's fatality rate significantly decreased.

Transportation events and contact with objects/equipment events were the most frequent fatal events in the oil and gas extraction industry, which is consistent with previously reported data (*7*,*8*). This analysis showed the rate of fatalities caused by contact with objects/equipment experienced the greatest decrease during 2003–2013 ($p<0.001$), which might be related to the increased use of automated technologies on drilling rigs such as hydraulic catwalks to move drill pipe from ground level to the rig floor and powered tongs used to make and break drilling pipe connections. A recent study found lower non-fatal injury rates on rigs with automated technologies designed to reduce workers' exposure to hazardous equipment (*9*). This report also found that the transportation-related fatality rate decreased significantly ($p<0.05$) despite an increase in the number of fatalities. Previous research showed the majority of transportation fatalities were the result of motor vehicle crashes killing occupants of light trucks (e.g., pickup trucks), which are largely unregulated (*8*). Transportation fatalities did not include deaths while commuting to and from work, as these are not typically considered work-related. However, frequent long distance commutes are common for workers in this industry and are an area of concern.

Collaboration between industry, government, and academic institutions might have contributed to improved safety for workers and likely should continue to drive the fatality rate further down. In 2003, the National Service, Transmission, Exploration and Production Safety Network was founded in South Texas by the Occupational Safety and Health Administration and industry to share best practices in oil and gas safety and health. Since then, the organization has expanded to 22 independent networks serving 15 oil and gas producing states. Another group, the National Occupational Research Agenda Oil and Gas Extraction Sector Council, was created by CDC in 2008 as a partnership program to establish an occupational safety and health research agenda. Since then, the council has created several safety products targeting high-risk workers and activities (*10*). In addition, regional groups, such as the Appalachian Shale Transportation Safety Workgroup, have formed to identify and share best practices in transportation safety.

This report is subject to at least three limitations. First, it would have been preferable to calculate fatality rates using estimates of the number of full-time equivalent workers, which takes overtime into consideration, but these estimates were not available for this industry. Second, changes made to the Occupational Injury and Illness Classification System for years 2011 and later are considered a break in series. Although event-type categories reported here did not undergo significant change in 2011, clarifications in the order of precedence for the event type categories were issued that might have led to differences in event coding starting in 2011. Lastly, fatal event numbers for 2013 are preliminary and might be incomplete. Historically, transportation event data are the most incomplete, and this could affect the trend.

Although the fatality rate in the oil and gas extraction industry remains an average of seven times higher than among U.S. workers in general (25.1 compared with 3.7 per 100,000 per year), the oil and gas extraction industry has achieved a substantial decrease in fatality rates in recent years. It is important for oil and gas industry employers to continue implementation of safety measures that target causes of the most frequent fatal events. One example is having a land transportation safety policy that outlines safety procedures for all workers who drive as a part of their duties. Another example is adoption of automated technologies that reduce workers' exposure to oil rig hazards. Occupational safety and health researchers need to continue and enhance surveillance efforts and identify risk factors for different types of fatal injuries among different sectors of the oil and gas extraction industry. The data from surveillance efforts will be useful to industry safety and health networks and can be used to create targeted interventions to reduce worker fatalities.

What is already known on this topic?

Fatality rates for workers in the oil and gas extraction industry have historically been higher than the rate for all workers (an average of seven times higher every year since 2003, when fatality rates for oil and gas workers were first added to the data collected). During 2003–2013, an oil and gas boom occurred and the industry doubled its workforce and experienced a 71% increase in active drilling rigs. Although the number of fatal injuries also increased during this time, trends in fatality rates during this boom have not been previously reported.

What is added by this report?

The fatality rate for the oil and gas extraction industry decreased by 36.3% ($p<0.001$) during 2003–2013, from 29.0 to 19.1 per 100,000 workers per year. The rate for fatalities caused by contact with objects and equipment experienced the greatest decrease (60.8%, $p<0.001$). Transportation incidents continue to be the leading cause of death.

What are the implications for public health practice?

It is important for oil and gas industry employers to continue to implement safety measures that target causes of the most frequent fatal events, including a land transportation safety policy for all workers who drive as a part of their duties. To target injury prevention programs, it is important that occupational safety and health researchers continue and enhance surveillance efforts to identify and report on risk factors for different types of fatal injuries among different sectors of the oil and gas extraction industry.

Acknowledgments

Census of Fatal Occupational Injuries Program, Bureau of Labor Statistics, U.S. Department of Labor; Devin Lucas, Western States Division, National Institute for Occupational Safety and Health, CDC.

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References

1. Bureau of Labor Statistics. Quarterly census of employment and wages. Washington, DC: US Department of Labor, Bureau of Labor Statistics; 2014. Available at http://data.bls.gov/cgi-bin/dsrv?en.
2. Baker Hughes, Inc. North American rig counts. Houston, TX: Baker Hughes; 2104. Available at http://phx.corporate-ir.net/phoenix.zhtml?c=79687&p=irol-reportsother.
3. Bureau of Labor Statistics. Injuries, illnesses, and fatalities: census of fatal occupational injuries (CFOI)—current and revised data. Washington, DC: US Department of Labor, Bureau of Labor Statistics; 2013. Available at http://www.bls.gov/iif/oshcfoi1.htm.
4. International Association of Oil & Gas Producers. Land transportation recommended practice. London: International Association of Oil & Gas Producers; 2014. Report no. 365 (Issue 2). Available at http://www.ogp.org.uk/pubs/365.pdf.
5. National Institute for Occupational Safety and Health. Preventing work-related motor vehicle crashes. Atlanta, GA: CDC, National Institute for Occupational Safety and Health; 2015. Pub. no. 2015–111. Available at http://www.cdc.gov/niosh/docs/2015-111/pdfs/2015-111.pdf.
6. American Petroleum Institute. Recommended practice for occupational safety for oil and gas well drilling and servicing operations. Washington, DC: American Petroleum Institute; 2015. Recommended practice No. 54.
7. CDC. Fatalities among oil and gas extraction workers—United States, 2003–2006. MMWR Morb Mortal Wkly Rep 2008;57:429–31.
8. Retzer KD, Hill RD, Pratt SG. Motor vehicle fatalities among oil and gas extraction workers. Accid Anal Prev 2013;51:168–74.
9. Blackley DJ, Retzer KD, Hubler WG, Hill RD, Laney AS. Injury rates on new and old technology oil and gas rigs operated by the largest United States onshore drilling contractor. Am J Ind Med 2014;57:1188–92.
10. National Institute for Occupational Safety and Health. Oil and gas extraction inputs: national occupational research agenda. Atlanta, GA: CDC, National Institute for Occupational Safety and Health; 2012. Available at http://www.cdc.gov/niosh/programs/oilgas/nora.html.

Acute Rheumatic Fever and Rheumatic Heart Disease Among Children — American Samoa, 2011–2012

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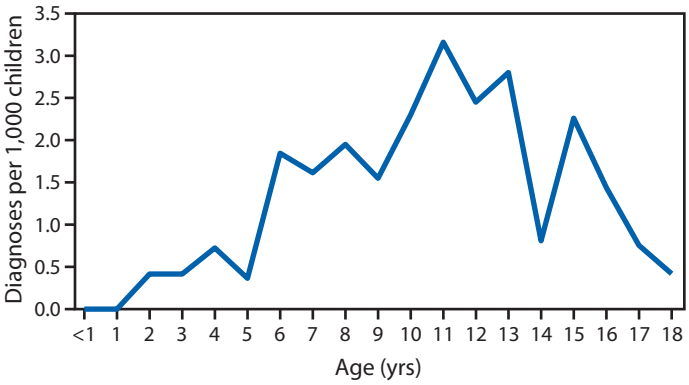
Acute rheumatic fever is a nonsuppurative, immune-mediated consequence of group A streptococcal pharyngitis (strep throat). Recurrent or severe acute rheumatic fever can cause permanent cardiac valve damage and rheumatic heart disease, which increases the risk for cardiac conditions (e.g., infective endocarditis, stroke, and congestive heart failure) (*1*,*2*). Antibiotics can prevent acute rheumatic fever if administered no more than 9 days after symptom onset. Long-term benzathine penicillin G (BPG) injections are effective in preventing recurrent acute rheumatic fever attacks and are recommended to be administered every 3–4 weeks for 10 years or until age 21 years to children who receive a diagnosis of acute rheumatic fever (*3*). During August 2013, in response to anecdotal reports of increasing rates of acute rheumatic fever and rheumatic heart disease, CDC collaborated with the American Samoa Department of Health and the Lyndon B. Johnson Tropical Medical Center (the only hospital in American Samoa) to quantify the number of cases of pediatric acute rheumatic fever and rheumatic heart disease in American Samoa and to assess the potential roles of missed pharyngitis diagnosis, lack of timely prophylaxis prescription, and compliance with prescribed BPG prophylaxis. Using data from medical records, acute rheumatic fever incidence was calculated as 1.1 and 1.5 cases per 1,000 children aged ≤18 years in 2011 and 2012, respectively; 49% of those with acute rheumatic fever subsequently received a diagnosis of rheumatic heart disease. Noncompliance with recommended prophylaxis with BPG after physician-diagnosed acute rheumatic fever was noted for 22 (34%) of 65 patients. Rheumatic heart disease point prevalence was 3.2 cases per 1,000 children in August 2013. Establishment of a coordinated acute rheumatic fever and rheumatic heart disease control program in American Samoa, likely would improve diagnosis, treatment, and patient compliance with BPG prophylaxis.

Acute rheumatic fever is no longer a nationally notifiable disease in the United States, and its annual incidence in the continental United States declined in the late 20th century to approximately 0.04–0.06 cases per 1,000 children (*4*). Exceptions to these low acute rheumatic fever incidence rates in the United States include Samoan persons living in Hawaii and residents of American Samoa, an American territory in the South Pacific (*5*,*6*). Acute rheumatic fever rates in Hawaii have been as high as nearly 0.1 cases per 1,000 children, with even higher rates among persons of Samoan and Hawaiian ethnicity (*5*). Acute rheumatic fever occurs most commonly among children aged 5–15 years.

Pediatric cases of acute rheumatic fever and rheumatic heart disease were defined as physician-diagnosed acute rheumatic fever or rheumatic heart disease among patients aged ≤18 years who had sought care during 2011–2012 at the hospital in American Samoa. *International Classification of Diseases, Ninth Revision* (ICD-9) codes and BPG prophylaxis registries including patients currently receiving BPG treatment at the hospital were used to identify cases of acute rheumatic fever and rheumatic heart disease during 2011–2012 and to estimate the August 2013 point prevalence of rheumatic heart disease. Acute rheumatic fever diagnostic criteria included classic "Jones criteria" until summer 2012 (*7*), after which more sensitive Australian and New Zealand guidelines for high-risk areas were used (*8*). Case finding for inpatients with diagnoses during 2011–2012 was conducted by using ICD-9 codes (390–398). In addition, hospital patient registries for BPG prophylaxis were reviewed to identify additional acute rheumatic fever and rheumatic heart disease patients. Duplicate cases were excluded. Medical records for all identified patients were reviewed to verify acute rheumatic fever or rheumatic heart disease diagnoses and BPG prophylaxis noncompliance, which included recorded missed or late doses. Case-finding using hospital BPG prophylaxis registries was conducted to determine the number of children known to be living with rheumatic heart disease at the time of the study. Acute rheumatic fever incidence (2011–2012) and rheumatic heart disease point prevalence (August 2013) were calculated by using 2010 U.S. Census Bureau data (American Samoa pop. = 55,519, including 24,652 persons aged ≤18 years).

Acute rheumatic fever incidence was 1.1 and 1.5 cases per 1,000 children, for 2011 and 2012, respectively. Of 65 children with physician-diagnosed acute rheumatic fever during 2011–2012, a total of 32 (49%) subsequently received a diagnosis of rheumatic heart disease. Acute rheumatic fever patients were predominantly male (60%); median age at acute rheumatic fever diagnosis was 11 years (range: 2–18 years) (Figure). The 41 patients with available data were of Polynesian (98%) or Fijian (2%) origin. Twelve (18%) patients had a diagnosis of

FIGURE. Average annual rate of acute rheumatic fever diagnoses per 1,000 children, by age — American Samoa, 2011–2012



pharyngitis noted in the medical record during the 6 weeks preceding acute rheumatic fever or rheumatic heart disease diagnosis. Noncompliance with post–acute rheumatic fever prophylaxis with BPG was noted for 22 (34%) patients.

Among 32 rheumatic heart disease patients with data, 21 (66%) received a diagnosis of rheumatic heart disease without a previous acute rheumatic fever diagnosis noted in the medical record, indicating that certain patients did not seek care or did not receive a diagnosis until after the disease had progressed. The point prevalence of rheumatic heart disease was 3.2 cases per 1,000 children in August 2013. Of 34 pharyngitis diagnoses made during 2011–2012 and reviewed in acute rheumatic fever patient records, three (9%) were made using rapid antigen detection testing, 15 (44%) were made using throat culture, and 16 (47%) were made without any diagnostic testing.

Discussion

In addition to causing pharyngitis, pyoderma, and severe invasive disease (e.g., streptococcal toxic shock syndrome and necrotizing fasciitis), group A streptococcal organisms can trigger postinfection syndromes that result from a crossreaction between patient antibodies to bacterial surface proteins and cardiac, neuronal, and synovial tissues (*9*). Acute rheumatic fever, characterized primarily by carditis, chorea, and polyarthritis, occurs a minimum of 2–3 weeks after an episode of untreated or inadequately treated pharyngitis. Acute rheumatic fever does not cause lasting damage to the nervous tissue or joints. However, damage to heart valves can be irreversible and is worsened by repeat episodes of acute rheumatic fever (*1*,*3*). Permanent valvular damage, or rheumatic heart disease, increases the risk for infective endocarditis, stroke, heart failure, and premature death, and might necessitate valve replacement surgery (*2*). Because pharyngitis and acute rheumatic fever are most common in children, the recurrence of acute rheumatic fever, and, thus, the risk for developing rheumatic heart disease, can continue into adolescence and young adulthood.

This investigation highlights a long-standing disparity in the acute rheumatic fever and rheumatic heart disease rates between children in American Samoa and children in the continental United States. In August 2013, rheumatic heart disease point prevalence in American Samoa (3.2 per 1,000 children) was approximately 10 times that previously estimated for industrialized countries (0.3 per 1,000 children) (*2*). With improved diagnosis and treatment of group A streptococcal pharyngitis, the United States and other industrialized countries have seen a steep decline in rheumatic heart disease prevalence since the mid-20th century. However, in some parts of the world, rheumatic heart disease is the most common cardiac disease of children and young adults (*3*). The highest rheumatic heart disease rates occur in sub-Saharan Africa, with an estimated 5.7 cases per 1,000 children aged 5–14 years, and in the Pacific region and indigenous populations of Australia and New Zealand, with 3.5 cases per 1,000 (*2*).

Multiple factors influence rates of acute rheumatic fever and rheumatic heart disease, including host immune factors and lifestyle (e.g., crowding or access to health care), as well as the biologic characteristics of circulating group A streptococcal strains (*1*). However, opportunities for prevention exist and include improving access to medical care and using evidence-based strategies to identify and treat group A streptococcal pharyngitis early (primary prevention) and diagnose and prevent recurrent acute rheumatic fever and rheumatic heart disease (secondary prevention) (*3*).

The World Health Organization recommends community-based acute rheumatic fever and rheumatic heart disease control programs, which include penicillin prophylaxis after an acute rheumatic fever diagnosis to prevent recurrent acute rheumatic fever and rheumatic heart disease (*1*). Coordinated control programs increase acute rheumatic fever and rheumatic heart disease awareness among patients and the community, improve coverage and compliance with penicillin prophylaxis and medical care, and decrease the rate of recurrent disease (*3*). Current programs are diverse in their delivery and complexity and include patient registries maintained by health care personnel, community-based prophylaxis, monitoring of medical needs (e.g., echocardiography appointments) and prophylaxis compliance, and education about the importance of prompt diagnosis of group A streptococcal pharyngitis (*3*). Programs in other countries have been shown to reduce morbidity, disability, and mortality from acute rheumatic fever and rheumatic heart disease (*1*). Before the decline in acute rheumatic fever

incidence in the United States, certain states had prioritized streptococcal disease control and managed control programs.

The morbidity typically associated with rheumatic heart disease, and the disparity between rates in American Samoa and the continental United States, warrant discussion of coordinated control and mandatory public health reporting of acute rheumatic fever and rheumatic heart disease cases in American Samoa. A rheumatic heart disease control program ideally would be operated with local staff members and include measures demonstrated to be successful in controlling acute rheumatic fever and rheumatic heart disease in other high-risk areas, with particular emphasis on timely diagnosis and treatment of group A streptococcal pharyngitis (*3*). In American Samoa, families often choose traditional remedies over medical care, and this study found that few patients with acute rheumatic fever had a recent diagnosis of pharyngitis. In addition, hospital physicians often rely on clinical, rather than laboratory, diagnosis of pharyngitis. Although penicillin prophylaxis is the only proven cost-effective secondary rheumatic heart disease prevention method, education of health care providers about adherence to clinical practice guidelines for pharyngitis diagnosis and treatment is crucial for acute rheumatic fever and rheumatic heart disease prevention (*10*).

The findings in this report are subject to at least three limitations. First, this study is likely affected by ascertainment bias, because it only reports acute rheumatic fever patients who sought care at the hospital. Those using traditional remedies for acute rheumatic fever symptoms and patients with mild disease might not seek care. Second, despite multiple case-finding modalities (i.e., registries and medical billing), physicians at the hospital do not assign ICD-9 codes and certain acute rheumatic fever diagnoses might have been missed by the coding staff. The pediatric BPG registry included only currently treated patients. Patients treated during 2011–2012 might have been removed from the registry because of death or emigration. In addition, if not in the adult registry, patients who transitioned from the pediatric to adult medicine service might have been lost to follow-up, and although the hospital serves the majority of residents, a limited number of persons might go off-island for health care. Therefore, this report likely underestimates the number of cases of pediatric acute rheumatic fever and rheumatic heart disease in American Samoa. Finally, medical records were not reviewed for concordance with acute rheumatic fever and rheumatic heart disease diagnostic criteria, potentially affecting the sensitivity and specificity of case ascertainment.

What is already known on this topic?

Inadequately treated group A streptococcal pharyngitis can lead to development of acute rheumatic fever and subsequent rheumatic heart disease, both of which are found at high rates among children living in the South Pacific. Long-term penicillin injections are effective in preventing recurrent acute rheumatic fever attacks and subsequent development of rheumatic heart disease.

What is added by this report?

This report describes a continued high incidence of acute rheumatic fever and prevalence of rheumatic heart disease in American Samoa. In August 2013, rheumatic heart disease point prevalence (3.2 per 1,000 children) was approximately 10 times that estimated for industrialized countries. The report also highlights the extent to which missed diagnoses, missed opportunities for treatment, and treatment noncompliance might contribute to the high rate of rheumatic heart disease.

What are the implications for public health practice?

Efforts to improve pharyngitis diagnosis and treatment and compliance with penicillin prophylaxis might reduce the burden of acute rheumatic fever and rheumatic heart disease among children in American Samoa. These goals might be effectively met by establishment of a coordinated disease control program.

Rheumatic heart disease is expected to cause considerable lifelong morbidity in American Samoa, where it is approximately 10 times more common than in the continental United States. Recommendations to curb rheumatic heart disease in American Samoa are manifold, including improving pharyngitis diagnosis and treatment with concurrent efforts to improve patient compliance with BPG prophylaxis. These goals might be met efficiently and cost-effectively by establishment of a coordinated acute rheumatic fever and rheumatic heart disease control program.

Acknowledgments

Pam Faumuina, Akapusi Ledua, MBBS, Lyndon B. Johnson Tropical Medical Center, American Samoa; Joseph Tufa, MBBS, Sharmain Mageo, Sai Fuimaono, MBBS, American Samoa Department of Health; Mary Dott, MD, Office of Public Health Preparedness and Response, CDC.

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References

1. World Health Organization. Rheumatic fever and rheumatic heart disease. World Health Organ Tech Rep Ser 2004;923:1–122.
2. Carapetis JR, Steer AC, Mulholland EK, Weber M. The global burden of group A streptococcal diseases. Lancet Infect Dis 2005;5:685–94.
3. Carapetis JR, Brown A, Wilson NJ, Edwards KN; Rheumatic Fever Guidelines Writing Group. An Australian guideline for rheumatic fever and rheumatic heart disease: an abridged outline. Med J Aust 2007;186:581–6.
4. Stockmann C, Ampofo K, Hersh AL, et al. Evolving epidemiologic characteristics of invasive group A streptococcal disease in Utah, 2002–2010. Clin Infect Dis 2012;55:479–87.
5. Chun LT, Reddy DV, Yim GK, Yamamoto LG. Acute rheumatic fever in Hawaii: 1966 to 1988. Hawaii Med J 1992;51:206–11.
6. Erdem G, Dodd A, Tuua A, et al. Acute rheumatic fever in American Samoa. Pediatr Infect Dis J 2007;26:1158–9.
7. Special Writing Group of the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease of the Council on Cardiovascular Disease in the Young of the American Heart Association. Guidelines for the diagnosis of rheumatic fever. Jones criteria, 1992 update. JAMA 1992;268:2069–73.
8. National Heart Foundation of Australia, Cardiac Society of Australia and New Zealand. Diagnosis and management of acute rheumatic fever and rheumatic heart disease in Australia—an evidence-based review. Sydney, Australia: National Heart Foundation of Australia; 2006. Available at http://doctor-ru.org/main/1800/1805.pdf.
9. Martin JM, Green M. Group A *Streptococcus*. Semin Pediatr Infect Dis 2006;17:140–8.
10. Shulman ST, Bisno AL, Clegg HW, et al. Clinical practice guideline for the diagnosis and management of group A streptococcal pharyngitis: 2012 update by the Infectious Diseases Society of America. Clin Infect Dis 2012;55:1279–82.

Notes from the Field

Outbreak of Skin Lesions Among High School Wrestlers — Arizona, 2014

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Skin infections are a common problem among athletes at all levels of competition; among wrestlers, 8.5% of all adverse events are caused by skin infections (*1*). Wrestlers are at risk because of the constant skin-to-skin contact required during practice and competition. The most common infections transmitted among high school wrestlers include fungal infections (e.g., ringworm), the viral infection herpes gladiatorum caused by herpes simplex virus–1 (HSV-1), and bacterial infections (e.g., impetigo) caused by *Staphylococcus* or *Streptococcus* species, including methicillin-resistant *Staphylococcal aureus* (MRSA) (*2*). On February 7, 2014, the Maricopa County Department of Public Health was notified of multiple wrestlers who reported skin lesions 2 weeks after participating in a wrestling tournament at school A. The tournament was held on January 24–25 and included 168 wrestlers representing 24 schools. The county health department initiated an investigation to identify cases of skin lesion, determine lesion etiology, identify risks associated with lesion development, and provide guidance for preventing additional cases.

Questionnaires were distributed to all wrestlers on teams that participated in the tournament and reported at least one skin lesion in a team member following the tournament. Medical records were obtained to verify lesion diagnosis where available. To include persons infected before and after the tournament, probable cases were defined as one or more skin lesions reported during January 1–March 1, 2014, by a wrestler who competed on a team that participated in the school A tournament. A confirmed case was a probable case with a physician-diagnosed skin lesion or laboratory-confirmation of a bacterial or viral infection of the skin.

A total of 47 cases (37 confirmed) were identified. Impetigo was the most common reported physician diagnosis (17 cases [46%]), followed by HSV-1 infection (11 [30%]), tinea corporis (two [5%]), and MRSA (two [5%]). One wrestler with physician-diagnosed HSV-1 reported having lesion onset 4 days before the January tournament and wrestling in the tournament with uncovered arm lesions. During the 2–9 days after the tournament, seven athletes who had wrestled in the tournament developed HSV-1 infection; during the 5–14 days after the tournament, three teammates who had not wrestled developed HSV-1. Another wrestler with physician-diagnosed impetigo reported having wrestled in the school A tournament with uncovered lesions on the head and neck. Subsequently, eight wrestlers who had participated in the tournament experienced impetigo 3–14 days after the tournament, and four teammates who did not participate in the tournament experienced impetigo 5–10 days after the tournament.

The Maricopa County Department of Public Health recommended that 1) wrestlers with visible, uncovered lesions be excluded from competition, 2) wrestling mats be disinfected between each match with a disinfectant approved by the Environmental Protection Agency as effective against MRSA and HSV-1, and 3) hand sanitizer be provided for use by all wrestlers during practices and competitions. In addition to implementing these recommendations, the Arizona Interscholastic Association also provided third-party clinicians who performed skin checks on each wrestler before competing.

This outbreak was caused by coincident spread of two distinct skin pathogens among high school wrestlers who had participated in the school A tournament. HSV-1 and impetigo caused by *Staphylococcus* or *Streptococcus* species were likely spread during the school A tournament by wrestlers who competed with uncovered lesions. CDC, the National Athletic Trainers' Association, and the National Federation of State and High School Associations have each released statements and guidelines providing athletic staff and players with education regarding skin lesion prevention, lesion identification, and management (*3*,*4*). The *Journal of the American Osteopathic Association* also has published an evidence-based review with return-to-play guidelines for common dermatologic infections among athletes (*5*). This outbreak highlights the need for athletes, their coaches, and athletic directors to follow well-established infection control guidelines, including keeping all skin lesions covered with a clean, dry dressing, and excluding athletes from competitions when lesions cannot remain covered.

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References

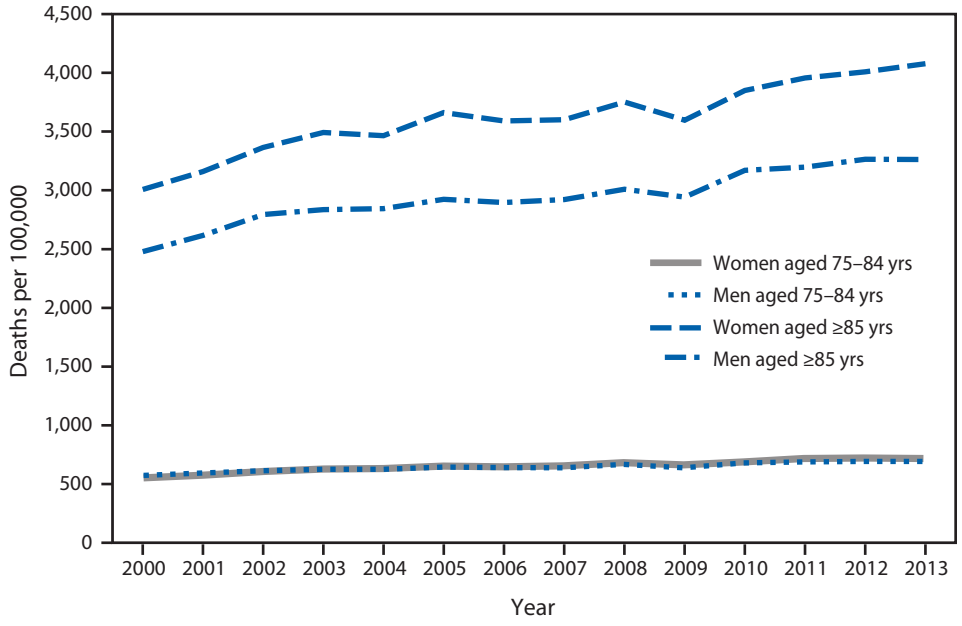
1. Yard EE, Collins CL, Dick RW, Comstock RD. An epidemiologic comparison of high school and college wrestling injuries. Am J Sports Med 2008;36:57–64.
2. Turbeville SD, Cowan LD, Greenfield RA. Infectious disease outbreaks in competitive sports: a review of the literature. Am J Sports Med 2006;34:1860–5.

3. National Federation of State High School Associations. General guidelines for sports hygiene, skin infections and communicable diseases. Indianapolis, IN: National Federation of State High School Associations; 2014. Available at http://www.nfhs.org/sports-resource-content/general-guidelines-for-sports-hygiene-skin-infections-and-communicable-diseases/.
4. CDC. MRSA information for coaches, athletic directors, and team healthcare providers: 5 steps to take if you think an athlete might have a skin infection. Atlanta, GA: US Department of Health and Human Services, CDC; 2013. Available at http://www.cdc.gov/mrsa/community/team-hc-providers/index.html.
5. Likness LP. Common dermatologic infections in athletes and return-to-play guidelines. J Am Osteopath Assoc 2011;111:373–9.

QuickStats

FROM THE NATIONAL CENTER FOR HEALTH STATISTICS

Death Rates* from Dementia† Among Persons Aged ≥75 Years, by Sex and Age Group — United States, 2000–2013



* Per 100,000 population.
† Deaths from dementia include underlying and contributing causes of death coded F01 (vascular dementia), F03 (unspecified dementia) or G30 (Alzheimer's disease) according to the *International Classification of Diseases, 10th Revision*.

During 2000–2013, death rates for dementia per 100,000 population increased for both men and women among persons aged 75–84 years and ≥85 years. Among persons aged 75–84 years, the rate increased 21% for men and 31% for women. Among persons aged ≥85 years, the rate increased 32% for men and 36% for women. Among persons aged ≥85 years, death rates were higher for women than men throughout the period, with death rates 25% higher among women than men in 2013 (4,077.4 versus 3,261.6 per 100,000 population).

Source: National Vital Statistics System. Multiple cause of death data, 2000–2013. Available at http://wonder.cdc.gov/mcd-icd10.html.

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ISSN: 0149-2195