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.
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.
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*. 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. 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.

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-}

\[\text{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.}
\[\text{Id. at 58-59.}
\[\text{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. In several studies, the levels of NNN and NNK in cigars and little cigars are equal to or exceed the levels in combustible cigarettes.

Polycyclic Aromatic Hydrocarbons or PAHs are formed when any organic matter is not completely burned. 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. 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.

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. In fact, smokers who switch to cigar smoking have little benefit in terms of mortality. 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. 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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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).


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).

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, prohibiting the use of modified risk terms including “light” and “low tar,” and imposing significant advertising and marketing restrictions. However, the Tobacco Control Act deferred any regulation of cigars to the future. 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. 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. 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. 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. Cigar manufacturers were also able to exploit CHIPRA by converting “small cigars” to “large cigars” by adding sepiolite to increase cigar weight, moving

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14 Id. § 387k(b)(2)(A)(ii).
15 See, e.g., 21 C.F.R. § 1140.1 – 1140.34 (2018).
19 Id.
20 Id. This particular change only required manufacturers to change their packaging, see id.
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. 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.

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. 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. 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.

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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


\(^{28}\) 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).

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


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

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

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. 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. In reality,

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. 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. 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. 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

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

42 Id.
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. 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. 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. 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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45 Id.
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.\footnote{Centers for Disease Control and Prevention, \textit{Use of Tobacco Tax Stamps to Prevent and Reduce Illicit Tobacco Trade — United States, 2014}, \textit{64 Morbidity and Mortality Weekly Report} 547 (2015), https://www.cdc.gov/mmwr/pdf/wk/mm6420.pdf.}

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.\footnote{U.S. v. Philip Morris, Inc., 449 F. Supp. 2d 1 (D.D.C. 2006), \textit{aff'd in relevant part}, 566 F.2d 1095 (D.C. Cir. 2009), \textit{cert. denied}, 130 S.Ct. 3501 (2010).}

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.\footnote{Id.}

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...
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,

[Signatures]

Joelle Lester
Director

Desmond Jenson
Senior Staff Attorney
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-(methylnitrosamo)-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-(methylnitrosamo)-1-(3-pyridyl)-1-butanol (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-Martín 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-(methylnitrosamo)-1-(3-pyridyl)-1-butanol [NNK], 4-(methylnitrosamo)-4-(3-pyridyl) butanal [NNA], N’-nitrosonornicotine [NNN], N’-nitrosodimethylamine [NAB], N’-nitrosodimethylamine [NAT], 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanol [NNAL], 4-(methylnitrosamo)-4-(3-pyridyl)-1-butanol [isoNNAL], and 4-(methylnitrosamo)-4-(3-pyridyl) butanoic acid [isoNNAC] (Hecht and Tricker 1999). isoNNAL and isoNNAC 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 (Boylan 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 tobacco are virtually free of TSNA (Green and Rodgman 1996; Caldwell and Conner 1990; Parsons et al. 1986; Spiegelhalder and Bartsch 1996; Brunnenmann et al. 1982; Fischer et al. 1990). It is recognized that TSNA are formed 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 (Peel 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 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 (≤ 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; Adikofier 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 P450 for the expression of genotoxicity. NNK metabolism by a P450-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.)
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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 when compared 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
\( \pm \text{SD} \) in the whole ETS surrogate and gas-phase ETS surrogate exposure atmospheres was 3.9 \( \pm \) 3.5 and 0.29 \( \pm \) 0.28 \( \mu \text{g/m}^3 \), 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 \( \mu \text{g} \) of NAT, 158 \( \mu \text{g} \) of NNN, and 135 \( \mu \text{g} \) of NNK per typical U.S. market full flavor cigarette (Chepiga et al. 2000); the total of all three TSNA would be about 475 \( \mu \text{g} \) 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.

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>A/J Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total NNK Dose (mg/25 gram mouse) required to induce significant incidence of lung tumors</td>
<td>Minimum Dose</td>
</tr>
<tr>
<td></td>
<td>0.52 mg</td>
</tr>
<tr>
<td>Total NNK Dose (mg/kg body weight) required to induce significant incidence of lung tumors</td>
<td>20.8 mg</td>
</tr>
<tr>
<td>NNK/Cigarette (Chepiga, 2000)</td>
<td>135 ng</td>
</tr>
<tr>
<td>Total Cigarette Equivalence/kg Mouse Body Weight</td>
<td>~154,000</td>
</tr>
<tr>
<td>Equivalent Dose of NNK in a 70 kg Smoker</td>
<td>1456 mg</td>
</tr>
<tr>
<td>Equivalent Number of Cigarettes</td>
<td>10,785,185</td>
</tr>
<tr>
<td>Comparison to Smoker (packs/day for 40 yrs)</td>
<td>37 packs</td>
</tr>
<tr>
<td>Comparison to Smoker (Years of smoking 2 packs/day)</td>
<td>739 years</td>
</tr>
</tbody>
</table>
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(< 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 35 ng NNK/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 methylyating 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 [5-3H]NNK binding of radioactivity (pyridyloxobutylolation) to rat hemoglobin (Kutner 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; Kutner 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 P450, 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\(^\alpha\)-methylguanine in A/J mouse lung DNA (Brown et al. 1999; Peterson and Hecht 1991). O\(^\alpha\)-MeG is a promutagenic base that induces guanine (G) to adenine (A) transition (Ronai et al. 1993). Any inhibition of the P\(_{450}\)–mediated \(\alpha\)-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\(^\alpha\)-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 \(\mu\)mol/mouse, sufficient to induce significant levels of O\(^\alpha\)-MeG adducts) resulted in a significant dose-dependent reduction in NNK-induced lung and liver O\(^\alpha\)-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 \(\mu\)mol/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 \(\alpha\)-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. 2001).
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Figure 2. Dose-dependent reduction of \(\text{O}^6\)-MeG concentration by IR4F cigarette smoke in A/J mice. Mice received a one-time, nose-only inhalation exposure of IR4F 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 \(\text{O}^6\)-MeG formation. The dosing of NNK (7.5 \(\mu\)mol/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 \(\text{O}^6\)-MeG by HPLC. (Mean ± SE; \(n = 18\); * = \(p < 0.05\)). (From Brown et al. 1999.)

An initial hypothesis of these studies was that urinary (S)-hydroxy acid could be a potential urinary biomarker of NNK and NNN \(\alpha\)-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 \(\mu\)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 \(\alpha\)-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 \(\alpha\)-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
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Figure 3. Effect of Kentucky reference 1R4F cigarette smoke (0.6 mg WTPM/L) on the lung and liver concentrations of O6-MeG in NNK-treated A/J mice. Mice received a once-time, nose-only inhalation regimen of either HEPA-filtered and humidified air (control) or 1R4F cigarette smoke at the previously determined MNLD (0.5 mg WTPM/L) for 2 h to monitor the effect of cigarette smoke on the concentration of O6-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 O6-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 \( (K_m = 0.5 \mu M; V_{max} = 388 \text{ fmol/min/mg protein and } K_m = 39; V_{max} = 21380) \), with a lower potential to form methylating and/or pyridoxal-butyllating 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 pyridoxal-butyllation undetectable in smokers' lungs (Blomeke et al. 1996). At a plausible level of NNK exposure, \( \alpha \)-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 \( (K_m = 0.6; V_{max} = 254 \text{ and } K_m = 44; V_{max} = 11340) \) (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-butanol (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 TSNAs and other 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 TSNAs in tobacco products? Sure it is, to the extent possible. Reduced TSNAs in tobacco products will result in reduced exposure to TSNAs. Will such reduction mean a reduced cancer risk? That cannot be determined until smokers have used reduced TSNAs 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 TSNAs cigarettes is similar in toxicity, mutagenicity, and carcinogenicity to CSC or smoke from cigarettes with current levels of TSNAs.

Based on our review of the published literature, we conclude that there is neither direct nor convincing evidence that NNK or TSNAs in smoke 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 TSNAs in tobacco smoke will have a significant impact on the lung cancer risks associated with cigarette smoking.

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Tobacco-Specific Nitrosamines and Tobacco Carcinogenesis


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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 times result 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.

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; φv, effective air exchange rate (1/τv) [L/hr]; φw, ventilatory air exchange rate (1/τw) [L/hr]; φd, particle deposition rate (1/τd) [L/hr]; τ, residence time (the time required for z(t) to reach 1/e times the original level) [hr]; a, air changes per hour [1/hr]; acm, air changes per minute [1/hr]; 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]; s, seconds [T]; min, minutes [T]; h, hours [T]; PM_{1.6}, 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]; τ, time-averaged average pollutant concentration [M/L^3]; g(t), total emission rate at time t [M/L^3]; τ\bar{z}, time-averaged emission rate [M/L^3]; \bar{z}, average emission rate per source [M/L^3]; τ\bar{ρ}, time-averaged number of sources; T\bar{z}, duration of cigar smoking [T]; q, total emissions [M]; q_{m}, emissions per unit mass smoked [M/M]; g_{eq}, equilibrium emission rate [M/L^3]; z_{eq}, equilibrium pollutant concentration [M/L^3]; t_{eq}, time required to reach equilibrium [T]; [T^*]=time units; [M^*]=mass units; [L^*]=length units; n=unit dimension (1, 2, or 3).

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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.
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 $\phi$, the ventilatory air exchange rate $\phi_V$, and the particle deposition rate $\phi_D$ can be determined for

![Figure 1](image-url)
<table>
<thead>
<tr>
<th>ID</th>
<th>Experiment description</th>
<th>Study duration, $T$ [min]</th>
<th>Cigar duration, $T_{c}$ [min]</th>
<th>Average CO concentration, $z$ [ppm]</th>
<th>Peak CO concentration, $z(T_{c})$ [ppm]</th>
<th>Average CO emission rate, $q$ CO emissions per cigar, $q$, $m$ [g]</th>
<th>Mass smoked, $m$ [g]</th>
<th>CO emissions per mass smoked, $q$, $m$ [mg/g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-1</td>
<td>1 Sante Fe Fairmont cigar (4/5/96); var $\phi_v$</td>
<td>424</td>
<td>18</td>
<td>3.6</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>O-2</td>
<td>1 Sante Fe Fairmont cigar (4/6/96); $\phi_v=4.5$ ach; see Figures 1 and 2</td>
<td>157</td>
<td>7.8</td>
<td>1.7</td>
<td>16</td>
<td>140</td>
<td>1.1 g</td>
<td>6</td>
</tr>
<tr>
<td>O-3</td>
<td>1 Sante Fe Fairmont cigar (4/7/96); $\phi_v=0.12$ ach</td>
<td>1284</td>
<td>24</td>
<td>7.5</td>
<td>19</td>
<td>46</td>
<td>1.1 g</td>
<td>6.1</td>
</tr>
<tr>
<td>O-4</td>
<td>1 Sante Fe Fairmont cigar (4/8/96); $\phi_v=2.1$ ach</td>
<td>339</td>
<td>20</td>
<td>1.8</td>
<td>15</td>
<td>61</td>
<td>1.2 g</td>
<td>6</td>
</tr>
<tr>
<td>O-5</td>
<td>1 Imported Ashton cigar (4/9/96); $\phi_v=1.8$ ach</td>
<td>275</td>
<td>28</td>
<td>2.5</td>
<td>15</td>
<td>42</td>
<td>1.2 g</td>
<td>14.3</td>
</tr>
<tr>
<td>O-6</td>
<td>1 Swisher Sweets cigar (4/22/96); var $\phi_v$</td>
<td>167</td>
<td>20</td>
<td>4.4</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>O-7</td>
<td>1 Swisher Sweets cigar (4/23/96); $\phi_v=0.96$ ach</td>
<td>450</td>
<td>42</td>
<td>2.4</td>
<td>9.5</td>
<td>23</td>
<td>980 mg</td>
<td>5.6</td>
</tr>
<tr>
<td>O-8</td>
<td>1 Dutch Masters El Presidente cigar (4/24/96); $\phi_v=1.9$ ach</td>
<td>220</td>
<td>15</td>
<td>5.7</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>O-9</td>
<td>1 Dutch Masters El Presidente cigar (4/25/96); $\phi_v=2.4$ ach</td>
<td>240</td>
<td>54</td>
<td>2.1</td>
<td>7.5</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>O-10</td>
<td>1 Dutch Masters El Presidente cigar (4/26/96); $\phi_v=0.06$ ach</td>
<td>1440</td>
<td>9</td>
<td>9.2</td>
<td>18</td>
<td>84</td>
<td>750 mg</td>
<td>6.6</td>
</tr>
<tr>
<td>O-11</td>
<td>1 AYC Grenadiers cigar (4/27/96); $\phi_v=0.12$ ach</td>
<td>1539</td>
<td>10</td>
<td>4.9</td>
<td>13</td>
<td>86</td>
<td>860 mg</td>
<td>4.9</td>
</tr>
<tr>
<td>O-12</td>
<td>1 AYC Grenadiers cigar (4/28/96); $\phi_v=3.0$ ach</td>
<td>130</td>
<td>17</td>
<td>1.7</td>
<td>7.0</td>
<td>37</td>
<td>630 mg</td>
<td>5.0</td>
</tr>
<tr>
<td>O-13</td>
<td>1 AYC Grenadiers cigar (4/29/96); $\phi_v=4.5$ ach</td>
<td>195</td>
<td>12</td>
<td>0.94</td>
<td>8.0</td>
<td>65</td>
<td>780 mg</td>
<td>4.9</td>
</tr>
<tr>
<td>O-14</td>
<td>1 AYC cigar (4/29/96); var $\phi_v$</td>
<td>87</td>
<td>17</td>
<td>0.89 (1.0 mg/m)</td>
<td>4.5</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>O-15</td>
<td>1 AYC cigar (4/30/96); $\phi_v=0.72$ ach</td>
<td>630</td>
<td>11</td>
<td>1.3</td>
<td>8.0</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

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 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 \( \phi \) is equal to \( \phi_V \) when obtained from the decay of CO (no surface sorption) and equal to \( \phi_{P+N} + \phi_V \) when obtained from the decay of RSP, which adheres to surfaces.

In real situations, the room will have a characteristic mixing time \( \tau_{\text{mix}} \), which is the time it takes for the pollutant to become well-mixed after the cigar is ignited. The decay rate \( \phi \) (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 \( t = \tau_{\text{mix}} \) (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 (referred to 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 \( \phi_V \) 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 operation 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 (referred to 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 Santana and a Paul Garmirian) and a second volunteer smoked a Marlboro Regular cigarette. The parlor of the residence was then monitored in order to measure the concentration of CO and RSP.

### Table 2. Physical characteristics of seven different cigars and Marlboro cigarettes.

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

\(^a\)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\(^b\) from two cigar experiments and one cigarette experiment in a 97 m\(^3\) parlor of a residence.

<table>
<thead>
<tr>
<th>ID</th>
<th>Experiment description(^b)</th>
<th>Study duration, (T) [min]</th>
<th>Source duration, (T_s) [min]</th>
<th>Average concentration, (\overline{\tau})</th>
<th>Peak concentration(^a), (\tau(T_s))</th>
<th>Average emission rate(^a), (\overline{q})</th>
<th>Emissions per cigar(^a), (m) [g]</th>
<th>Mass smoked(^a), (q_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-1</td>
<td>1 Santona cigar smoked by a person (3/1/97); (\phi = 2.0) ach</td>
<td>300 (5 h)</td>
<td>76 (1.3 h)</td>
<td>0.96 ppm CO (1.1 mg/m(^3))</td>
<td>3.0 ppm CO</td>
<td>14 mg CO/min</td>
<td>1.1 g CO</td>
<td>8.8</td>
</tr>
<tr>
<td>R-2</td>
<td>1 Paul Garmirian cigar smoked by a person (3/9/97); (\phi_s = 0.9) ach; (\phi = 1.2) ach for RSP; (\phi = 1.5) ach for PAH</td>
<td>285 (4.75 h)</td>
<td>90 (1.5 h)</td>
<td>0.16 mg/m(^3) RSP; 0.55 (\mu)g/m(^3) PAH</td>
<td>0.35 mg/m(^3) RSP</td>
<td>0.98 mg RSP/min; 0.0042 mg PAH/min</td>
<td>88 mg RSP; 0.38 mg PAH</td>
<td>10.8</td>
</tr>
<tr>
<td>R-3</td>
<td>1 Marlboro cigarette smoked by a person (3/9/97); (\phi = 1.3) ach for RSP; (\phi = 2.0) ach for PAH</td>
<td>127 (2.75 h)</td>
<td>9</td>
<td>0.065 mg/m(^3) RSP; 0.33 (\mu)g/m(^3) PAH</td>
<td>0.16 mg/m(^3) RSP</td>
<td>1.9 mg RSP/min; 0.015 mg PAH/min</td>
<td>17 mg RSP; 0.14 mg PAH</td>
<td>0.4</td>
</tr>
</tbody>
</table>

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 $\phi = 2.0$ and $\phi = 0.9$ 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](image-url)  

**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.

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**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-

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**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). Formulas for these three quantities, respectively, are as follows:

\[
\bar{g}_c = \bar{z} \phi / \bar{n}
\]

\[
q = \bar{g} T = \bar{g}_c \bar{n} T = \bar{z} \phi v T
\]

\[
q_m = q / m
\]

where \( \bar{g}_c \) is the average pollutant emission rate [mg/min], \( \bar{z} \) is the average pollutant concentration [mg/m³], \( \phi \) is the effective air exchange rate or decay rate [ach], \( \bar{n} \) is the volume of the room [m³], \( \bar{n} \) 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

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**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 \( \phi \) (see above discussion), the room volume \( V \), and the average pollutant emission rate, \( \bar{E}_e \), 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 \( T_e \) 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 \( T_e=8–76 \text{ min} \). There is

![Figure 5. Theoretical curves demonstrating how the pollutant peak airborne mass is affected by the cigar duration \( T_e \) and pollutant decay rate \( \phi \). 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.](image-url)
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 ($\phi=0.5–10$ 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 $\phi$ 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: $x_{eq}=g_{eq}/\nu \phi$. 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 $g_c$ ranging from 14 to 140 mg/min and total emissions

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**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 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 \( \phi \). 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 \( (T = 180 \text{~min}) \) and decay rate \( (\phi = 1 \text{ or } 5 \text{~ach}) \), 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 \( (14 \text{~mg/min}) \) and the AyC and Dutch Master cigars having the lowest total emissions \( (630–860 \text{~mg/cigar}) \). 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 \( (82 \text{~mg/g}) \) and the Dutch Masters \( (114 \text{~mg/g}) \) have the smallest. Although the Ashton has one of the lowest emission rates and the smallest mass-normalized emissions, its large \( (14.3 \text{~g}) \) causes its total emissions to be as high as any of the other cigars that were studied \( (1.2 \text{~g}) \).

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 \( (43 \text{ and } 0.34 \text{~mg/g}) \), and RSP and PAH emission rates that were two and four times as large as the cigar \( (1.9 \text{ and } 0.015 \text{~mg/min}) \). However, the total RSP and PAH emissions of the cigarette are five and three times higher, respectively, than those of the cigarette \( (17 \text{ and } 0.14 \text{~mg}) \), 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 \( (e.g., 0.55 \text{~g}) \); 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 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 \( (40 \text{ versus } 8 \text{~mg/g}) \), and the PAH emissions for a cigarette can be about ten times that of a cigar \( (0.34 \text{ versus } 0.035 \text{~mg/g}) \). Leaderer and Hammond (1991) report 48 mg/g of RSP emitted from a cigar \( (a \text{ cigarillo according to Leaderer, personal communication}) \) 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.

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A comparative study of the mutagenicity of various types of tobacco products

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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.

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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...
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 nonmalignant 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 TSNAs 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 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. (alkla 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 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.

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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 IS2 reference dry snuff (NCSU, 2006). The presence of glycerin and menthol in both low-moisture products is also atypical of the IS2. 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 Spier- to, 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 (kretex) 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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight (mg/unit)</th>
<th>Puff count (per unit)</th>
<th>MS TPM (mg/unit)</th>
<th>CO (mg/unit)</th>
<th>Water (mg/unit)</th>
<th>Nicotine (mg/unit)</th>
<th>Tar (mg/unit)</th>
<th>Propylene glycol (mg/unit)</th>
<th>Glycerol (mg/unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
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<td>Mean SD</td>
</tr>
<tr>
<td>Flue-cured cigarette</td>
<td>1112</td>
<td>10</td>
<td>10.7</td>
<td>0.5</td>
<td>24.6</td>
<td>1.8</td>
<td>15.5</td>
<td>0.4</td>
<td>4.24</td>
</tr>
<tr>
<td>Clove cigarette</td>
<td>789</td>
<td>12</td>
<td>7.45</td>
<td>0.19</td>
<td>11.0</td>
<td>0.0</td>
<td>6.80</td>
<td>0.2</td>
<td>0.76</td>
</tr>
<tr>
<td>Dark tobacco cigarette</td>
<td>969</td>
<td>6</td>
<td>6.54</td>
<td>0.13</td>
<td>12.6</td>
<td>0.6</td>
<td>14.6</td>
<td>0.6</td>
<td>1.00</td>
</tr>
<tr>
<td>CIII-7</td>
<td>972</td>
<td>5</td>
<td>7.99</td>
<td>0.21</td>
<td>14.6</td>
<td>0.4</td>
<td>12.4</td>
<td>0.3</td>
<td>1.53</td>
</tr>
<tr>
<td>KR2R4F</td>
<td>1076</td>
<td>5</td>
<td>8.79</td>
<td>0.25</td>
<td>10.9</td>
<td>0.3</td>
<td>12.1</td>
<td>0.5</td>
<td>0.80</td>
</tr>
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<td>Cigarillo 1</td>
<td>1075</td>
<td>33</td>
<td>11.6</td>
<td>0.4</td>
<td>49.2</td>
<td>3.3</td>
<td>52.3</td>
<td>1.7</td>
<td>8.32</td>
</tr>
<tr>
<td>Cigarillo 2</td>
<td>1077</td>
<td>9</td>
<td>12.0</td>
<td>0.7</td>
<td>58.2</td>
<td>3.8</td>
<td>52.7</td>
<td>2.9</td>
<td>12.0</td>
</tr>
<tr>
<td>Bidi 1</td>
<td>371</td>
<td>16</td>
<td>13.9</td>
<td>1.8</td>
<td>50.1</td>
<td>8.7</td>
<td>19.5</td>
<td>2.3</td>
<td>11.8</td>
</tr>
<tr>
<td>Bidi 2</td>
<td>459</td>
<td>8</td>
<td>16.4</td>
<td>2.5</td>
<td>43.2</td>
<td>12.7</td>
<td>18.1</td>
<td>3.9</td>
<td>11.3</td>
</tr>
<tr>
<td>Cigar 1</td>
<td>3415</td>
<td>199</td>
<td>35.8</td>
<td>1.1</td>
<td>229</td>
<td>32</td>
<td>NM</td>
<td>NM</td>
<td>64.4</td>
</tr>
<tr>
<td>Cigar 2</td>
<td>4309</td>
<td>51</td>
<td>50.5</td>
<td>9.3</td>
<td>215</td>
<td>11</td>
<td>NM</td>
<td>NM</td>
<td>63.6</td>
</tr>
<tr>
<td>Pipe tobacco 1</td>
<td>1501</td>
<td>1</td>
<td>80.3</td>
<td>25.0</td>
<td>150</td>
<td>21</td>
<td>NM</td>
<td>NM</td>
<td>52.5</td>
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<tr>
<td>Pipe tobacco 2</td>
<td>1501</td>
<td>1</td>
<td>82.9</td>
<td>49.8</td>
<td>198</td>
<td>25</td>
<td>NM</td>
<td>NM</td>
<td>73.5</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Country of origin</th>
<th>Menthol (mg/g) Mean SD</th>
<th>Nicotine (mg/g) Mean SD</th>
<th>Glycerol (mg/g) Mean SD</th>
<th>Dry matter (%) Mean SD</th>
<th>Moisture (%) Mean SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
</tr>
<tr>
<td>Tableted low-moisture snuff</td>
<td>US</td>
<td>5.94</td>
<td>0.07</td>
<td>5.71</td>
<td>0.34</td>
<td>0.314</td>
</tr>
<tr>
<td>Low-moisture wintergreen snuff</td>
<td>US</td>
<td>6.44</td>
<td>0.15</td>
<td>9.17</td>
<td>0.06</td>
<td>0.561</td>
</tr>
<tr>
<td>Gutkha</td>
<td>India</td>
<td>7.60</td>
<td>0.18</td>
<td>2.10</td>
<td>0.11</td>
<td>1.97</td>
</tr>
<tr>
<td>Long-cut fruit-flavored moist snuff</td>
<td>US</td>
<td>BDL</td>
<td>BDL</td>
<td>14.3</td>
<td>0.1</td>
<td>BDL</td>
</tr>
<tr>
<td>Pouched moist snuff</td>
<td>US</td>
<td>BDL</td>
<td>BDL</td>
<td>13.3</td>
<td>0.4</td>
<td>BDL</td>
</tr>
<tr>
<td>Zarda chewing tobacco</td>
<td>India</td>
<td>21.8</td>
<td>0.5</td>
<td>26.7</td>
<td>1.0</td>
<td>46.3</td>
</tr>
</tbody>
</table>

BDL means below detection limit.

- All data reported on an “as is” basis; Means and standard deviations presented are from three sample replicates.
- The menthol, nicotine, and glycerol contents of the products were estimated from analysis of the DMSO extracts.
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.
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, slope values in terms of revertants per milligram of nicotine

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nicotine dose range µg/plate</th>
<th>Replicate 1</th>
<th>Replicate 2</th>
<th>Replicate 3</th>
<th>All replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flue-cured cigarette</td>
<td>0–8</td>
<td>10,505</td>
<td>606</td>
<td>11,028</td>
<td>299</td>
</tr>
<tr>
<td>Clove cigarette</td>
<td>0–8</td>
<td>8274</td>
<td>700</td>
<td>9115</td>
<td>270</td>
</tr>
<tr>
<td>Dark tobacco cigarette</td>
<td>0–4</td>
<td>21,400</td>
<td>1027</td>
<td>14,609</td>
<td>1198</td>
</tr>
<tr>
<td>CIM-7</td>
<td>0–9</td>
<td>7709</td>
<td>407</td>
<td>7130</td>
<td>514</td>
</tr>
<tr>
<td>KR2R4F</td>
<td>0–9</td>
<td>11,934</td>
<td>1123</td>
<td>10,464</td>
<td>779</td>
</tr>
<tr>
<td>Cigarillo 1</td>
<td>0–6</td>
<td>14,066</td>
<td>756</td>
<td>15,673</td>
<td>626</td>
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<tr>
<td>Cigarillo 2</td>
<td>0–6</td>
<td>14,809</td>
<td>575</td>
<td>17,580</td>
<td>1035</td>
</tr>
<tr>
<td>Bidi 1</td>
<td>0–5</td>
<td>11,407</td>
<td>656</td>
<td>11,092</td>
<td>553</td>
</tr>
<tr>
<td>Bidi 2</td>
<td>0–7</td>
<td>10,172</td>
<td>491</td>
<td>8050</td>
<td>833</td>
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<tr>
<td>Cigar 1</td>
<td>0–5</td>
<td>15,597</td>
<td>641</td>
<td>13,937</td>
<td>344</td>
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<tr>
<td>Cigar 2</td>
<td>0–4</td>
<td>21,764</td>
<td>1041</td>
<td>22,431</td>
<td>1089</td>
</tr>
<tr>
<td>Pipe tobacco 1</td>
<td>0–4</td>
<td>9425</td>
<td>874</td>
<td>10,395</td>
<td>656</td>
</tr>
<tr>
<td>Pipe tobacco 2</td>
<td>0–4</td>
<td>12,935</td>
<td>793</td>
<td>13,031</td>
<td>1283</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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<th></th>
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<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tableted low-moisture snuff</td>
<td>0–8</td>
<td>US</td>
<td>6270</td>
<td>990</td>
<td>4760</td>
<td>661</td>
<td>4202</td>
<td>560</td>
<td>5077</td>
<td>463</td>
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<tr>
<td>Low-moisture wintergreen snuff</td>
<td>0–13</td>
<td>US</td>
<td>1421</td>
<td>394</td>
<td>1369</td>
<td>705</td>
<td>2801</td>
<td>194</td>
<td>1863</td>
<td>354</td>
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<td>Gutkha</td>
<td>0–3</td>
<td>India</td>
<td>13,902</td>
<td>985</td>
<td>4163</td>
<td>995</td>
<td>7577</td>
<td>1300</td>
<td>8547</td>
<td>1714</td>
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<tr>
<td>Long-cut fruit-flavored moist snuff</td>
<td>0–20</td>
<td>US</td>
<td>1120</td>
<td>146</td>
<td>1585</td>
<td>112</td>
<td>1182</td>
<td>151</td>
<td>1296</td>
<td>119</td>
</tr>
<tr>
<td>Pouched moist snuff</td>
<td>0–19</td>
<td>US</td>
<td>1615</td>
<td>170</td>
<td>1990</td>
<td>128</td>
<td>2265</td>
<td>172</td>
<td>1957</td>
<td>117</td>
</tr>
<tr>
<td>Zarda chewing tobacco</td>
<td>0–37</td>
<td>India</td>
<td>554</td>
<td>84</td>
<td>286</td>
<td>206</td>
<td>663</td>
<td>138</td>
<td>501</td>
<td>94</td>
</tr>
</tbody>
</table>

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

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 of 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 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.
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
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 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 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 also 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


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

<table>
<thead>
<tr>
<th>Sample</th>
<th>TA98+S9 revertants per milligram Nicotine</th>
<th>TA100+S9 revertants per milligram Nicotine</th>
<th>Ratio TA98+S9/TA100+S9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flue-cured cigarette</td>
<td>19,265</td>
<td>10,921</td>
<td>1.764</td>
</tr>
<tr>
<td>Clove cigarette</td>
<td>19,619</td>
<td>8376</td>
<td>2.342</td>
</tr>
<tr>
<td>Dark tobacco cigarette</td>
<td>70,024</td>
<td>18,250</td>
<td>3.837</td>
</tr>
<tr>
<td>CIM-7</td>
<td>15,940</td>
<td>7633</td>
<td>2.088</td>
</tr>
<tr>
<td>KR2R4F</td>
<td>33,681</td>
<td>10,613</td>
<td>3.174</td>
</tr>
<tr>
<td>Cigarillo 1</td>
<td>51,771</td>
<td>13,989</td>
<td>3.701</td>
</tr>
<tr>
<td>Cigarillo 2</td>
<td>53,810</td>
<td>15,188</td>
<td>3.543</td>
</tr>
<tr>
<td>Bidi 1</td>
<td>22,343</td>
<td>11,440</td>
<td>1.953</td>
</tr>
<tr>
<td>Bidi 2</td>
<td>19,346</td>
<td>9905</td>
<td>1.953</td>
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<tr>
<td>Cigarette 1</td>
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<td>Cigarette 2</td>
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</tr>
<tr>
<td>Bidis 1</td>
<td>19,346</td>
<td>9905</td>
<td>1.953</td>
</tr>
<tr>
<td>Bidis 2</td>
<td>22,343</td>
<td>11,440</td>
<td>1.953</td>
</tr>
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<td>19,265</td>
<td>10,921</td>
<td>1.764</td>
</tr>
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<td>Nickle 13</td>
<td>19,619</td>
<td>8376</td>
<td>2.342</td>
</tr>
<tr>
<td>Dark tobacco</td>
<td>70,024</td>
<td>18,250</td>
<td>3.837</td>
</tr>
<tr>
<td>CIM-7</td>
<td>15,940</td>
<td>7633</td>
<td>2.088</td>
</tr>
<tr>
<td>KR2R4F</td>
<td>33,681</td>
<td>10,613</td>
<td>3.174</td>
</tr>
<tr>
<td>Cigarillo 1</td>
<td>51,771</td>
<td>13,989</td>
<td>3.701</td>
</tr>
<tr>
<td>Cigarillo 2</td>
<td>53,810</td>
<td>15,188</td>
<td>3.543</td>
</tr>
<tr>
<td>Bidi 1</td>
<td>22,343</td>
<td>11,440</td>
<td>1.953</td>
</tr>
<tr>
<td>Bidi 2</td>
<td>19,346</td>
<td>9905</td>
<td>1.953</td>
</tr>
<tr>
<td>Cigarette 1</td>
<td>42,061</td>
<td>13,967</td>
<td>3.011</td>
</tr>
<tr>
<td>Cigarette 2</td>
<td>64,867</td>
<td>21,586</td>
<td>3.005</td>
</tr>
<tr>
<td>Bidis 1</td>
<td>19,346</td>
<td>9905</td>
<td>1.953</td>
</tr>
<tr>
<td>Bidis 2</td>
<td>22,343</td>
<td>11,440</td>
<td>1.953</td>
</tr>
</tbody>
</table>


NCI, 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.


Health Risks Associated With Cigar Smoking

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.

Cigars have increased rapidly in recent years, coincident with the aggressive glamorization and promotion of cigars. \(^1\) 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”\(^2\); 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.

**TABLE 1**

<table>
<thead>
<tr>
<th>Type of Tobacco</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigars</td>
<td>12 to 20% sun-cured leaf tobacco with other tobacco leaves and filler.</td>
</tr>
<tr>
<td>Cigarettes</td>
<td>60% sun-cured leaf tobacco with 20%-40% air-cured leaf tobacco and filler.</td>
</tr>
</tbody>
</table>

**Author Affiliations:** Behavioral Research Center (Drs Baker and Dye and Mr Ainsworth and Ms Crammer), Epidemiology and Surveillance Research (Dr Thun), American Cancer Society, Atlanta, Ga; American Health Foundation, Valhalla, NY (Dr Hoffmann); Repace Associates Inc, Bowie, Md (Mr Repace) and Pinney Associates Inc (Dr Henningfield and Mr Pinney), and Smoking and Tobacco Control Program, National Cancer Institute (Mr Shopland), Bethesda, Md; Department of Medicine, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, New Brunswick (Dr Slade); Tobacco Control Policies Project (Mr Shanks) and University of California at San Diego School of Medicine (Dr Burns), University of California, San Diego; and Massachusetts Department of Public Health, Boston (Dr Connolly).

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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 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(103-104) 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(103-104)

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.2 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 Survey7 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 Aficionado*). Electronic and print media report America’s “rediscovery” of the premium cigar smoked by the affluent and successful members of society.1(105-106,109) The sale of cigars has expanded from tobacco stores,

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**Table 1. Cigar Types and Characteristics**

<table>
<thead>
<tr>
<th>Classification System</th>
<th>Cigar</th>
<th>Weight, g</th>
<th>Length, mm</th>
<th>Diameter, mm</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>US Department of the Treasury2†</td>
<td>Small</td>
<td>≤1.36</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>&gt;1.36</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>US Federal Trade Commission3†</td>
<td>Little</td>
<td>&lt;1.36</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>1.36-4.54</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>Large</td>
<td>&gt;4.54</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Hoffman and Hoffman,1(105-106) 1998</td>
<td>Little</td>
<td>0.9-1.3</td>
<td>70-100</td>
<td>...</td>
<td>Shaped like cigarettes; some with filter tips</td>
</tr>
<tr>
<td></td>
<td>Small</td>
<td>1.3-2.5</td>
<td>70-120</td>
<td>...</td>
<td>Also known as cigarillos, cheroots; usually have wood or plastic mouthpieces</td>
</tr>
<tr>
<td></td>
<td>Regular</td>
<td>5-17</td>
<td>110-150</td>
<td>≤17</td>
<td>Usually rolled to a tip on at least 1 end and banded; machine made or hand rolled</td>
</tr>
<tr>
<td></td>
<td>Premium</td>
<td>≤22</td>
<td>127-214</td>
<td>12-23</td>
<td>Almost all are hand rolled; currently cost from US $4 to &gt;$20</td>
</tr>
</tbody>
</table>

*Ellipses indicate none specified.
†Converted from the US Customary System of measurement to the International System for comparability.
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. 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. 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. Compared with nonsmokers, cigar smokers also underestimate the cancer risk of exposure to environmental cigar smoke.

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 traditional image of sports figures smoking cigars at a victory celebration.

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. 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. 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.

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. 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. At least 63 of these compounds are known to be carcinogenic, including 11 known human carcinogens. 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. 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 exhibits greater tumorigenic activity in mice compared with the tar from cigarettes, because cigar tar contains higher concentrations of carcinogenic polycyclic aromatic hydrocarbons.

Environmental Tobacco Smoke

Sidestream smoke (the aerosol emitted from the burning cone of a cigar, cigarette, or pipe during the interval be-
between puffs and the portion of the inhaled smoke that is not retained and is exhaled25 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(pp105-158)

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 μg/m³ on a 24-hour average).1(pp161-179,27) 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(pp105-130,25,26,31) 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(pp105-130) 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,2 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 I (CPS-1) of the American Cancer Society, a cohort study conducted between 1959 and 1972.1(pp105-158) Of the 442455 white male subjects in CPS-1, 15191 were primary cigar smokers and had never smoked cigarettes, 7404 were secondary cigar smokers and had previously smoked cigarettes, 10300 were mixed smokers and currently smoking both cigars and cigarettes; and 175000 were cigarette-only smokers. The cancer risks for these groups were compared with rates for 92300 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 113000 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 underscores 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 underscores 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.

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

N J Wald, H C Watt

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 achieving the lower risk of pipe and cigar smokers who switch to cigars or pipes rather than cigarettes is uncertain. We used data from the three specified 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 IL128 co-oximeter, as described in detail previously, for all men at the time they attended the medical centre. 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 cigarettes 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>
<thead>
<tr>
<th>Smoking group</th>
<th>No of men</th>
<th>No of pipes, cigars, and cigarettes</th>
<th>Weight of tobacco (g)</th>
<th>Observed carboxyhaemoglobin saturation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipe/cigar smoker, never smoked cigarettes (non-switchers):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipes only</td>
<td>472</td>
<td>6 (2-16)</td>
<td>12.1 (4.0-20.3)</td>
<td>1.4 (0.7-3.6)</td>
</tr>
<tr>
<td>Cigars only</td>
<td>651</td>
<td>2 (0-6)</td>
<td>4.7 (2.0-17.8)</td>
<td>0.9 (0.5-2.4)</td>
</tr>
<tr>
<td>Both</td>
<td>186</td>
<td>4 (2-12)</td>
<td>11.5 (5.5-24.9)</td>
<td>1.0 (0.5-2.6)</td>
</tr>
<tr>
<td>Either or both</td>
<td>1309</td>
<td>3 (0-11)</td>
<td>8.1 (2.1-20.3)</td>
<td>1.0 (0.6-3.2)</td>
</tr>
<tr>
<td>Pipe/cigar smoker, switched from cigarettes over 20 years ago (switchers):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipes only</td>
<td>187</td>
<td>7 (3-15)</td>
<td>12.1 (4.0-20.3)</td>
<td>1.9 (0.7-4.8)</td>
</tr>
<tr>
<td>Cigars only</td>
<td>212</td>
<td>2 (0-5)</td>
<td>5.9 (2.0-17.4)</td>
<td>1.0 (0.6-4.2)</td>
</tr>
<tr>
<td>Both</td>
<td>63</td>
<td>5 (2-13)</td>
<td>10.8 (5.0-22.4)</td>
<td>1.2 (0.5-3.8)</td>
</tr>
<tr>
<td>Either or both</td>
<td>522</td>
<td>4 (1-12)</td>
<td>8.1 (2.0-20.3)</td>
<td>1.2 (0.6-4.5)</td>
</tr>
<tr>
<td>Current cigarette smokers (cigarettes/day):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-14</td>
<td>947</td>
<td>8 (3-12)</td>
<td>8.9 (3.0-12.6)</td>
<td>1.8 (0.8-4.8)</td>
</tr>
<tr>
<td>15-24</td>
<td>1387</td>
<td>20 (15-20)</td>
<td>20.9 (15.0-20.5)</td>
<td>4.4 (2.0-7.0)</td>
</tr>
<tr>
<td>25-34</td>
<td>1133</td>
<td>30 (25-30)</td>
<td>30.3 (25.0-30.5)</td>
<td>5.7 (3.2-8.7)</td>
</tr>
<tr>
<td>≥35</td>
<td>717</td>
<td>40 (35-50)</td>
<td>49.3 (35.0-50.5)</td>
<td>9.2 (3.6-9.8)</td>
</tr>
<tr>
<td>All amounts</td>
<td>4184</td>
<td>20 (7-40)</td>
<td>20.9 (7.0-40.0)</td>
<td>4.6 (1.4-8.0)</td>
</tr>
</tbody>
</table>

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

| Smoking group                        | Self described inhaling category (% men in each smoking group) | Median carboxyhaemoglobin saturation by inhaling category (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipel/cigars smoker, never smoked cigarettes (non-switchers):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipes only</td>
<td>1 (41)</td>
<td>1.2 (0.6-4.5)</td>
</tr>
<tr>
<td>Cigars only</td>
<td>2 (78)</td>
<td>0.9 (0.5-2.4)</td>
</tr>
<tr>
<td>Both</td>
<td>5 (88)</td>
<td>1.1 (0.7-4.8)</td>
</tr>
<tr>
<td>Either or both</td>
<td>8 (1.0)</td>
<td>0.9 (0.5-2.6)</td>
</tr>
<tr>
<td>Pipe/cigars smoker, switched from cigarettes 20 years ago (switchers):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipes only</td>
<td>5 (18)</td>
<td>1.3 (0.8-4.6)</td>
</tr>
<tr>
<td>Cigars only</td>
<td>9 (33)</td>
<td>0.9 (0.5-2.4)</td>
</tr>
<tr>
<td>Both</td>
<td>15 (46)</td>
<td>1.1 (0.7-4.8)</td>
</tr>
<tr>
<td>Either or both</td>
<td>13 (40)</td>
<td>0.9 (0.5-2.6)</td>
</tr>
<tr>
<td>Current cigarette smoker (cigarettes/day):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-14</td>
<td>7 (23)</td>
<td>1.3 (0.8-4.6)</td>
</tr>
<tr>
<td>15-24</td>
<td>5 (18)</td>
<td>1.0 (0.6-4.2)</td>
</tr>
<tr>
<td>25-34</td>
<td>3 (10)</td>
<td>1.0 (0.5-2.6)</td>
</tr>
<tr>
<td>≥35</td>
<td>4 (14)</td>
<td>0.9 (0.5-2.6)</td>
</tr>
<tr>
<td>All amounts</td>
<td>5 (18)</td>
<td>1.0 (0.6-4.2)</td>
</tr>
</tbody>
</table>

BMJ VOLUME 314 28 JUNE 1997 1861

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

<table>
<thead>
<tr>
<th>Smoking group at entry</th>
<th>Total No of men</th>
<th>Ischaemic heart disease</th>
<th>Lung cancer</th>
<th>Chronic obstructive lung disease</th>
<th>All three diseases</th>
<th>All cause mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Relative mortality</td>
<td>No of deaths</td>
<td>Relative mortality</td>
<td>No of deaths</td>
<td>Relative mortality</td>
</tr>
<tr>
<td>Lifelong non-smoker</td>
<td>6539</td>
<td>1.09</td>
<td>125</td>
<td>1.00</td>
<td>7</td>
<td>1.00</td>
</tr>
<tr>
<td>Former cigarette smoker, stopped smoking over 20 years before entry</td>
<td>1465</td>
<td>1.05</td>
<td>59</td>
<td>1.01</td>
<td>3</td>
<td>†</td>
</tr>
<tr>
<td>Pipe/cigar smoker, never smoked cigarettes (non-switchers)</td>
<td>1399</td>
<td>0.98</td>
<td>33</td>
<td>3.19†</td>
<td>6</td>
<td>†</td>
</tr>
<tr>
<td>Pipe/cigar smoker, switched from cigarettes over 20 years before entry (switchers)</td>
<td>522</td>
<td>1.29</td>
<td>25</td>
<td>8.64†</td>
<td>9</td>
<td>†</td>
</tr>
<tr>
<td>Current cigarette smoker</td>
<td>4182</td>
<td>2.27*</td>
<td>193</td>
<td>16.4*</td>
<td>77</td>
<td>29.5*</td>
</tr>
</tbody>
</table>

*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, relative to mortality in four reference groups

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

*Risk in reference group set at 1.00. Relative mortality estimate can be derived by selecting entry in column of 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.

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). 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, 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 who gave up smoking altogether, and, of course, 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.

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.

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

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Conflict of interest: None.

Risk Perception and Cigar Smoking Behavior

Frank Baker, PhD; Joseph T. Dye, PhD; Maxine M. Denniston, MSPH; Stuart R. Ainsworth, MA

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. Nevertheless, stimulated by ample and effective advertising, in the last several years, cigar smoking has increased dramatically. The tobacco industry proudly reports an increase of 50% or more in the sale of premium cigars in the United States. The US Department of Agriculture estimates that cigar sales in the United States increased by almost 50% between 1993 and 1997. 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%. 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.

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. Studies have shown that people who continue smoking tend to consider their own risks from...
smoking to be lower than the risks of other smokers.\textsuperscript{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.”\textsuperscript{10} Weinstein\textsuperscript{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.\textsuperscript{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.

\textbf{METHOD}

\textbf{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.

\textbf{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.\textsuperscript{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
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 withpronounced 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-
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. 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 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Current Nonsmokers</th>
<th>Current Cigar Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Age**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-24</td>
<td>60</td>
<td>8.8</td>
</tr>
<tr>
<td>25-44</td>
<td>278</td>
<td>40.5</td>
</tr>
<tr>
<td>45-54</td>
<td>132</td>
<td>19.3</td>
</tr>
<tr>
<td>55-64</td>
<td>93</td>
<td>13.6</td>
</tr>
<tr>
<td>65 or more</td>
<td>122</td>
<td>17.8</td>
</tr>
<tr>
<td>Male**</td>
<td>326</td>
<td>47.1</td>
</tr>
<tr>
<td>Race/Ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>556</td>
<td>81.8</td>
</tr>
<tr>
<td>Black</td>
<td>73</td>
<td>10.7</td>
</tr>
<tr>
<td>Asian</td>
<td>12</td>
<td>1.8</td>
</tr>
<tr>
<td>Other</td>
<td>39</td>
<td>5.7</td>
</tr>
<tr>
<td>Hispanic</td>
<td>52</td>
<td>7.6</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than high school graduate</td>
<td>62</td>
<td>9.2</td>
</tr>
<tr>
<td>High school graduate</td>
<td>209</td>
<td>31.1</td>
</tr>
<tr>
<td>Some college</td>
<td>162</td>
<td>24.1</td>
</tr>
<tr>
<td>College graduate or higher</td>
<td>239</td>
<td>35.6</td>
</tr>
<tr>
<td>Income</td>
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<td></td>
</tr>
<tr>
<td>Less than $10,000</td>
<td>32</td>
<td>5.4</td>
</tr>
<tr>
<td>$10,000 – 24,999</td>
<td>103</td>
<td>17.5</td>
</tr>
<tr>
<td>$25,000 – 49,999</td>
<td>241</td>
<td>40.9</td>
</tr>
<tr>
<td>$50,000 or more</td>
<td>213</td>
<td>36.2</td>
</tr>
<tr>
<td>Occupation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Professional/managerial/owner</td>
<td>219</td>
<td>47.2</td>
</tr>
<tr>
<td>Sales, clerical</td>
<td>80</td>
<td>17.3</td>
</tr>
<tr>
<td>Craftsman/foreman</td>
<td>74</td>
<td>16.0</td>
</tr>
<tr>
<td>Semiskilled/unskilled</td>
<td>47</td>
<td>10.2</td>
</tr>
<tr>
<td>Service worker</td>
<td>42</td>
<td>9.1</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Marital Status**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Married/marriage-like relationship</td>
<td>428</td>
<td>62.1</td>
</tr>
<tr>
<td>Single, never married</td>
<td>132</td>
<td>19.2</td>
</tr>
<tr>
<td>Divorced/separated</td>
<td>76</td>
<td>11.0</td>
</tr>
<tr>
<td>Widowed</td>
<td>53</td>
<td>7.7</td>
</tr>
</tbody>
</table>

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

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**TABLE 3**

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

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

*** P-value of χ² median split test for difference in medians between current cigar smokers and current nonsmokers <.001
"typical cigar smokers" to be athletic (23 of 100) than did nonsmokers (18 of 100), although this difference was not statistically significant at the $\alpha = .01$ 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.²³ 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

**TABLE 4**

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

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

* P-value of $\chi^2$ median split test for difference in medians between current cigar smokers and current nonsmokers <.05
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. 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. 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's 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.

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 non-smokers 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 non-smokers 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


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.
Survey (NATS), 18.4% of adult cigarette users reported smoking little filtered cigars as their usual type smoked, while 61.8% reported smoking cigarillos. Nearly 60% of these adult cigarette smokers were also current or former cigarette smokers.4 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.6 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.8 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.14–17 Adults may endorse the perceived safety of LCCs because, unlike cigarettes, they are not addressed in anti-smoking media campaigns.14–20 Further, infrequent non-daily use14,21 and misperceptions about the constituents of LCCs, such as the belief that LCCs have “natural” or fewer harmful ingredients10,20,21 compared to cigarettes, may also under-score 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 30% of the survey items. A total of 5717 respondents were retained for analyses. Additional details about the parent study can be found elsewhere.21 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 138 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) compared to 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) compared to 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’ intention 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

<table>
<thead>
<tr>
<th>Sociodemographic characteristics</th>
<th>Overall (N = 1191)</th>
<th>Group 1: Dual smokers (n = 584)</th>
<th>Group 2: LCC-susceptible cigarette smokers (n = 117)</th>
<th>Group 3: Non-LCC-susceptible cigarette smokers (n = 490)</th>
<th>Group 1 vs. Group 3</th>
<th>Group 2 vs. Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking status</td>
<td>Overall</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>Adjusted RRR (95% CI)</td>
</tr>
<tr>
<td>Prevalence</td>
<td>47.2</td>
<td>12.7</td>
<td>40.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>52.5</td>
<td>65.4</td>
<td>47.6</td>
<td>38.8</td>
<td></td>
<td>0.337** (0.248-0.459)</td>
</tr>
<tr>
<td>Female</td>
<td>47.5</td>
<td>34.6</td>
<td>52.4</td>
<td>61.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18–29</td>
<td>21.7</td>
<td>24.5</td>
<td>34.6</td>
<td>14.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30–44</td>
<td>28.7</td>
<td>30.1</td>
<td>33.6</td>
<td>25.7</td>
<td></td>
<td>0.705 (0.417-1.194)</td>
</tr>
<tr>
<td>45–59</td>
<td>31.9</td>
<td>29.9</td>
<td>27.3</td>
<td>35.6</td>
<td></td>
<td>0.543* (0.331-0.889)</td>
</tr>
<tr>
<td>≥60</td>
<td>17.7</td>
<td>15.6</td>
<td>4.4</td>
<td>24.4</td>
<td></td>
<td>0.371** (0.220-0.627)</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White, non-Hispanic</td>
<td>63.2</td>
<td>69.1</td>
<td>44.0</td>
<td>62.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black, non-Hispanic</td>
<td>17.2</td>
<td>14.2</td>
<td>26.9</td>
<td>17.7</td>
<td></td>
<td>0.789 (0.504-1.235)</td>
</tr>
<tr>
<td>Other, non-Hispanic</td>
<td>6.4</td>
<td>5.1</td>
<td>14.7</td>
<td>5.4</td>
<td></td>
<td>0.692 (0.366-1.310)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>13.1</td>
<td>11.6</td>
<td>14.3</td>
<td>14.5</td>
<td></td>
<td>0.624 (0.362-1.075)</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than high school</td>
<td>22.2</td>
<td>20.1</td>
<td>32.1</td>
<td>21.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High school</td>
<td>36.1</td>
<td>33.8</td>
<td>29.3</td>
<td>41.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Some college</td>
<td>31.0</td>
<td>33.3</td>
<td>30.7</td>
<td>28.5</td>
<td></td>
<td>1.167 (0.731-1.864)</td>
</tr>
<tr>
<td>Bachelor's degree +</td>
<td>10.6</td>
<td>12.8</td>
<td>7.9</td>
<td>9.0</td>
<td></td>
<td>1.400 (0.777-2.523)</td>
</tr>
<tr>
<td>Income</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;$15 000</td>
<td>24.8</td>
<td>22.1</td>
<td>34.4</td>
<td>25.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$15 000 to $24 999</td>
<td>10.8</td>
<td>9.1</td>
<td>15.4</td>
<td>11.4</td>
<td></td>
<td>0.812 (0.502-1.313)</td>
</tr>
<tr>
<td>$25 000 to $39 999</td>
<td>20.2</td>
<td>19.7</td>
<td>18.2</td>
<td>21.3</td>
<td></td>
<td>1.039 (0.641-1.746)</td>
</tr>
<tr>
<td>$40 000 to $59 999</td>
<td>17.4</td>
<td>17.4</td>
<td>13.3</td>
<td>18.7</td>
<td></td>
<td>0.880 (0.539-1.438)</td>
</tr>
<tr>
<td>$60 000 to $84 999</td>
<td>11.9</td>
<td>15.6</td>
<td>5.9</td>
<td>9.4</td>
<td></td>
<td>1.440 (0.817-2.539)</td>
</tr>
<tr>
<td>$85 000 to $99 999</td>
<td>5.0</td>
<td>6.2</td>
<td>1.8</td>
<td>4.6</td>
<td></td>
<td>1.092 (0.518-2.304)</td>
</tr>
<tr>
<td>≥$100 000</td>
<td>9.8</td>
<td>9.8</td>
<td>10.9</td>
<td>9.5</td>
<td></td>
<td>0.849 (0.458-1.582)</td>
</tr>
<tr>
<td>Employment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not employed</td>
<td>49.8</td>
<td>46.3</td>
<td>53.9</td>
<td>52.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Employed</td>
<td>50.2</td>
<td>53.7</td>
<td>46.1</td>
<td>47.4</td>
<td></td>
<td>0.923 (0.663-1.285)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Risk Perceptions</th>
<th>Overall (N = 1191)</th>
<th>Group 1: Dual smokers (n = 584)</th>
<th>Group 2: LCC-susceptible cigarette smokers (n = 117)</th>
<th>Group 3: Non-LCC-susceptible cigarette smokers (n = 490)</th>
<th>Group 1 vs. Group 3</th>
<th>Group 2 vs. Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Become addicted to LCCs?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>4.6</td>
<td>5.5</td>
<td>7.2</td>
<td>2.8</td>
<td>Ref.</td>
<td>Ref.</td>
</tr>
<tr>
<td>Yes</td>
<td>73.1</td>
<td>76.2</td>
<td>52.1</td>
<td>76.1</td>
<td>1.720 (0.739–4.003)</td>
<td>2.448 (0.818–7.325)</td>
</tr>
<tr>
<td>I don’t know</td>
<td>22.3</td>
<td>18.3</td>
<td>40.7</td>
<td>21.1</td>
<td>1.048 (0.670–1.639)</td>
<td>2.829** (1.571–5.094)</td>
</tr>
<tr>
<td>Harmfulness of LCC smoking compared to cigarettes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less harmful</td>
<td>3.1</td>
<td>4.3</td>
<td>2.8</td>
<td>1.7</td>
<td>Ref.</td>
<td>Ref.</td>
</tr>
<tr>
<td>About the same</td>
<td>58.6</td>
<td>58.0</td>
<td>53.4</td>
<td>60.3</td>
<td>1.745 (0.680–4.476)</td>
<td>0.851 (0.198–3.658)</td>
</tr>
<tr>
<td>More harmful</td>
<td>13.9</td>
<td>16.9</td>
<td>8.2</td>
<td>12.1</td>
<td>1.533 (0.968–2.426)</td>
<td>0.814 (0.371–1.783)</td>
</tr>
<tr>
<td>I don’t know</td>
<td>24.4</td>
<td>20.8</td>
<td>33.6</td>
<td>25.9</td>
<td>1.318 (0.814–2.133)</td>
<td>0.936 (0.451–2.027)</td>
</tr>
<tr>
<td>Harmfulness of smoking LCCs daily</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very risky</td>
<td>42.4</td>
<td>42.6</td>
<td>26.8</td>
<td>46.9</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Somewhat risky</td>
<td>26.0</td>
<td>32.4</td>
<td>22.9</td>
<td>19.4</td>
<td>1.614* (1.083–2.405)</td>
<td>1.875 (0.880–3.994)</td>
</tr>
<tr>
<td>A little risky</td>
<td>8.6</td>
<td>8.3</td>
<td>18.3</td>
<td>6.1</td>
<td>0.915 (0.451–1.856)</td>
<td>3.136* (1.123–8.759)</td>
</tr>
<tr>
<td>Not at all risky</td>
<td>0.6</td>
<td>0.7</td>
<td>1.2</td>
<td>0.0</td>
<td>0.816 (0.105–6.296)</td>
<td>2.495 (0.265–23.457)</td>
</tr>
<tr>
<td>I don’t know</td>
<td>22.3</td>
<td>15.9</td>
<td>30.8</td>
<td>27.2</td>
<td>0.426* (0.211–0.857)</td>
<td>1.349 (0.488–3.727)</td>
</tr>
<tr>
<td>Harmfulness of smoking LCCs occasionally</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very risky</td>
<td>22.1</td>
<td>19.7</td>
<td>16.4</td>
<td>26.8</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Somewhat risky</td>
<td>29.3</td>
<td>31.4</td>
<td>25.7</td>
<td>28.0</td>
<td>1.339 (0.861–2.082)</td>
<td>1.182 (0.521–2.680)</td>
</tr>
<tr>
<td>A little risky</td>
<td>20.3</td>
<td>24.6</td>
<td>18.4</td>
<td>15.8</td>
<td>1.710* (1.019–2.870)</td>
<td>1.059 (0.420–2.670)</td>
</tr>
<tr>
<td>Not at all risky</td>
<td>4.7</td>
<td>5.5</td>
<td>7.7</td>
<td>2.8</td>
<td>2.277 (0.957–5.414)</td>
<td>2.324 (0.631–8.550)</td>
</tr>
<tr>
<td>I don’t know</td>
<td>23.6</td>
<td>18.8</td>
<td>31.8</td>
<td>26.6</td>
<td>1.681 (0.801–3.524)</td>
<td>0.839 (0.292–2.408)</td>
</tr>
</tbody>
</table>

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.
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 constituents26,27 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.

**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)**

<table>
<thead>
<tr>
<th>Risk perception</th>
<th>“Will smoke LCC again” vs. “Won’t smoke LCC again”</th>
<th>“I don’t know” vs. “Won’t smoke LCC again”</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adjusted RRR (95% CI)</td>
<td>Adjusted RRR (95% CI)</td>
</tr>
<tr>
<td></td>
<td>Ref.</td>
<td>Ref.</td>
</tr>
<tr>
<td>Become addicted to LCC?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4.831 (1.148–20.34)*</td>
<td>2.662 (0.652–10.86)</td>
</tr>
<tr>
<td>No</td>
<td>1.662 (0.795–3.475)</td>
<td>0.910 (0.415–1.993)</td>
</tr>
<tr>
<td>I don’t know</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harmfulness of LCC smoking compared to cigarette smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>About the same level of harm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less harmful</td>
<td>1.000 (0.313–3.199)</td>
<td>0.267 (0.0716–0.994)*</td>
</tr>
<tr>
<td>More harmful</td>
<td>0.875 (0.395–1.937)</td>
<td>0.431 (0.191–0.974)*</td>
</tr>
<tr>
<td>I don’t know</td>
<td>0.479 (0.208–1.102)</td>
<td>0.702 (0.292–1.687)</td>
</tr>
<tr>
<td>Harmfulness of smoking LCCs daily</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very risky</td>
<td>2.300 (1.102–4.802)*</td>
<td>2.395 (1.331–5.063)**</td>
</tr>
<tr>
<td>Somewhat risky</td>
<td>3.243 (0.990–10.62)</td>
<td>4.850 (1.877–12.53)**</td>
</tr>
<tr>
<td>A little risky</td>
<td>0.472 (0.0576–3.866)</td>
<td>—</td>
</tr>
<tr>
<td>I don’t know</td>
<td>6.788 (1.555–29.64)*</td>
<td>12.95 (3.956–42.39)*****</td>
</tr>
<tr>
<td>Harmfulness of smoking LCCs occasionally</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very risky</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Somewhat risky</td>
<td>1.019 (0.441–2.354)</td>
<td>0.892 (0.405–1.964)</td>
</tr>
<tr>
<td>A little risky</td>
<td>0.646 (0.237–1.760)</td>
<td>0.508 (0.217–1.189)</td>
</tr>
<tr>
<td>I don’t know</td>
<td>0.366 (0.0900–1.491)</td>
<td>0.602 (0.195–1.859)</td>
</tr>
</tbody>
</table>

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)**

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

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.
dual cigarette and LCC use may increase the likelihood of nicotine dependence,\textsuperscript{11,12} cancer,\textsuperscript{15,26,31} and other chronic health conditions.\textsuperscript{24,32}

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\textsuperscript{15} found that a lack of coverage of cigarettes 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\textsuperscript{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).\textsuperscript{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.\textsuperscript{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.\textsuperscript{35} 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,\textsuperscript{36} may be appropriate to address these misperceptions among adults.

**Funding**

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**Declaration of Interests**

None declared.

**References**

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

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 123.1% increase).

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).

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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...
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.

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

<table>
<thead>
<tr>
<th>Year</th>
<th>Cigarettes</th>
<th>All combustible tobacco</th>
<th>Noncigarette combustible tobacco</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total consumption (in millions)</td>
<td>% change</td>
<td>Adult per capita consumption (in millions)</td>
</tr>
<tr>
<td>2000</td>
<td>435,570</td>
<td>—</td>
<td>2,076</td>
</tr>
<tr>
<td>2001</td>
<td>426,720</td>
<td>-2.0</td>
<td>2,010</td>
</tr>
<tr>
<td>2002</td>
<td>415,724</td>
<td>-2.6</td>
<td>1,936</td>
</tr>
<tr>
<td>2003</td>
<td>400,327</td>
<td>-3.7</td>
<td>1,844</td>
</tr>
<tr>
<td>2004</td>
<td>397,655</td>
<td>-0.7</td>
<td>1,811</td>
</tr>
<tr>
<td>2005</td>
<td>381,098</td>
<td>-4.2</td>
<td>1,717</td>
</tr>
<tr>
<td>2006</td>
<td>380,594</td>
<td>-0.1</td>
<td>1,695</td>
</tr>
<tr>
<td>2007</td>
<td>361,590</td>
<td>-5.0</td>
<td>1,591</td>
</tr>
<tr>
<td>2008</td>
<td>346,419</td>
<td>-4.2</td>
<td>1,507</td>
</tr>
<tr>
<td>2009</td>
<td>317,736</td>
<td>-8.3</td>
<td>1,367</td>
</tr>
<tr>
<td>2010</td>
<td>300,451</td>
<td>-5.4</td>
<td>1,278</td>
</tr>
<tr>
<td>2011</td>
<td>292,769</td>
<td>-2.6</td>
<td>1,232</td>
</tr>
</tbody>
</table>

**% 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

<table>
<thead>
<tr>
<th>Year</th>
<th>Loose tobacco</th>
<th>Cigars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Roll-your-own* (in millions)</td>
<td>% change</td>
</tr>
<tr>
<td>2000</td>
<td>5,995</td>
<td>—</td>
</tr>
<tr>
<td>2001</td>
<td>4,714</td>
<td>-21.4</td>
</tr>
<tr>
<td>2002</td>
<td>5,737</td>
<td>21.7</td>
</tr>
<tr>
<td>2003</td>
<td>6,207</td>
<td>8.2</td>
</tr>
<tr>
<td>2004</td>
<td>6,600</td>
<td>6.4</td>
</tr>
<tr>
<td>2005</td>
<td>8,614</td>
<td>30.5</td>
</tr>
<tr>
<td>2006</td>
<td>8,594</td>
<td>-0.2</td>
</tr>
<tr>
<td>2007</td>
<td>9,326</td>
<td>8.5</td>
</tr>
<tr>
<td>2008</td>
<td>10,721</td>
<td>15.0</td>
</tr>
<tr>
<td>2009</td>
<td>6,006</td>
<td>-44.0</td>
</tr>
<tr>
<td>2010</td>
<td>3,168</td>
<td>-47.2</td>
</tr>
<tr>
<td>2011</td>
<td>2,622</td>
<td>-17.2</td>
</tr>
</tbody>
</table>

**% 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.
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.

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 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.
References


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, permitted 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. 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, 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.

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
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.

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**

References


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 infant’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 [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 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.

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.

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

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.

## Acknowledgment

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

## References


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


<table>
<thead>
<tr>
<th>Survey years</th>
<th>Total</th>
<th>U.S.-born</th>
<th>Foreign-born</th>
</tr>
</thead>
<tbody>
<tr>
<td>1982–1984</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1988–1994</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1999–2006</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2007–2010</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 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.


**Reported by:** Cheryl D. Fryar, MSPH, cfryar@cdc.gov, 301-458-4537.

Data presented by the Notifiable Disease Data Team and 122 Cities Mortality Data Team in the weekly *MMWR* are provisional, based on weekly reports to CDC by state health departments. Address all inquiries about the *MMWR* Series, including material to be considered for publication, to Editor, *MMWR* Series, Mailstop E-90, CDC, 1600 Cliffron Rd., N.E., Atlanta, GA 30333 or to mmwrq@cdc.gov.

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Reasons for Starting and Stopping Electronic Cigarette Use

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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...
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 Knowledge Panel 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).

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

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

Notes: <sup>a</sup> Smoked less than 100 cigarettes in lifetime; <sup>b</sup> Smoked 100 or more cigarettes in lifetime but does not currently smoke; <sup>c</sup> Among current smokers (n = 3328); <sup>d</sup> 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.
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).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number Who Stopped Using E-Cigarettes/Total Number in Category (Unweighted $n$ and Weighted %)</th>
<th>Bivariate</th>
<th>Multivariate (Includes Bivariate Correlates $p &lt; 0.05$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$ (%)</td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td><strong>Overall</strong></td>
<td>2281/3878 (65.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Participant</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (Ref)</td>
<td>849/1544 (62.7)</td>
<td>1.00 -</td>
<td>1.00 -</td>
</tr>
</tbody>
</table>
| 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.

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

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**


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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-(methylamino)-1-(3-pyridyl)-1-butanol and its glucuronides (total NNAL), metabolites of the tobacco-specific lung carcinogen 4-(methylamino)-1-(3-pyridyl)-1-butanol ((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 χ² 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 χ² 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; χ² = 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 x 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

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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

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References


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

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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.

<table>
<thead>
<tr>
<th>Demographics</th>
<th>0.3 mg nicotine cigarettes (n = 52)</th>
<th>0.05 mg nicotine cigarettes (n = 53)</th>
<th>Nicotine lozenges (n = 60)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>51 39.4 ± 14.0</td>
<td>53 40.7 ± 13.3</td>
<td>59 43.1 ± 12.4</td>
<td>0.3312</td>
</tr>
<tr>
<td>Female</td>
<td>26 50.0%</td>
<td>23 43.4%</td>
<td>29 48.3%</td>
<td>0.6967</td>
</tr>
<tr>
<td>Non-Hispanic whites</td>
<td>47 90.4%</td>
<td>44 83.0%</td>
<td>49 81.7%</td>
<td>0.7653</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
<td>0.8782</td>
</tr>
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<td>Less than high school graduate</td>
<td>2 3.9%</td>
<td>2 3.8%</td>
<td>3 5.0%</td>
<td></td>
</tr>
<tr>
<td>High school graduate</td>
<td>12 23.1%</td>
<td>11 20.8%</td>
<td>17 28.3%</td>
<td></td>
</tr>
<tr>
<td>Greater than high school graduate</td>
<td>37 71.2%</td>
<td>40 75.5%</td>
<td>39 65.0%</td>
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</tr>
<tr>
<td>Marital status</td>
<td></td>
<td></td>
<td></td>
<td>0.1052</td>
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<tr>
<td>Never married</td>
<td>22 42.3%</td>
<td>25 47.2%</td>
<td>22 36.7%</td>
<td></td>
</tr>
<tr>
<td>Currently married</td>
<td>22 42.3%</td>
<td>17 32.1%</td>
<td>16 26.7%</td>
<td></td>
</tr>
<tr>
<td>Currently not married</td>
<td>8 15.4%</td>
<td>11 20.8%</td>
<td>21 35.0%</td>
<td></td>
</tr>
<tr>
<td>Cigarettes per day</td>
<td>52 19.8 ± 7.8</td>
<td>53 21.1 ± 8.1</td>
<td>60 21.3 ± 9.6</td>
<td>0.6066</td>
</tr>
<tr>
<td>Duration of having smoked at this rate (years)</td>
<td>52 15.4 ± 13.0</td>
<td>53 14.1 ± 12.6</td>
<td>59 15.7 ± 14.1</td>
<td>0.7960</td>
</tr>
<tr>
<td>Age smoking first cigarette (years)</td>
<td>52 14.4 ± 2.9</td>
<td>53 15.5 ± 4.8</td>
<td>60 14.5 ± 2.9</td>
<td>0.2518</td>
</tr>
<tr>
<td>Age becoming a regular smoker (years)</td>
<td>52 16.5 ± 3.0</td>
<td>52 19.4 ± 7.3</td>
<td>58 17.7 ± 4.1</td>
<td>0.0195</td>
</tr>
<tr>
<td>Motivation to quit (0-10 scale)</td>
<td>52 9.0 ± 1.1</td>
<td>53 9.2 ± 1.0</td>
<td>59 9.2 ± 1.3</td>
<td>0.6928</td>
</tr>
<tr>
<td>Number of quit attempts</td>
<td></td>
<td></td>
<td></td>
<td>0.4482</td>
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<tr>
<td>1–2</td>
<td>13 25.0%</td>
<td>17 32.1%</td>
<td>13 21.7%</td>
<td></td>
</tr>
<tr>
<td>3–5</td>
<td>14 26.9%</td>
<td>14 26.4%</td>
<td>24 40.0%</td>
<td></td>
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<tr>
<td>6–10</td>
<td>12 23.1%</td>
<td>10 18.9%</td>
<td>13 21.7%</td>
<td></td>
</tr>
<tr>
<td>11+</td>
<td>10 19.2%</td>
<td>4 7.6%</td>
<td>9 15.0%</td>
<td></td>
</tr>
<tr>
<td>Spouse or significant other smokes</td>
<td>20 38.5%</td>
<td>20 37.7%</td>
<td>21 35.0%</td>
<td>0.9515</td>
</tr>
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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.

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Geometric mean (95% confidence interval)</th>
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<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>Total cotinine</td>
<td></td>
</tr>
<tr>
<td>0.3 mg cigarettes</td>
<td>4057 (3323, 4952)</td>
</tr>
<tr>
<td>0.05 mg cigarettes</td>
<td>4216 (3492, 5090)</td>
</tr>
<tr>
<td>Nicotine lozenge</td>
<td>3917 (3399, 4514)</td>
</tr>
<tr>
<td>Total NNAL</td>
<td></td>
</tr>
<tr>
<td>0.3 mg cigarettes</td>
<td>0.96 (0.73, 1.26)</td>
</tr>
<tr>
<td>0.05 mg cigarettes</td>
<td>0.92 (0.70, 1.21)</td>
</tr>
<tr>
<td>Nicotine lozenge</td>
<td>1.06 (0.84, 1.35)</td>
</tr>
<tr>
<td>Total NNN</td>
<td></td>
</tr>
<tr>
<td>0.3 mg cigarettes</td>
<td>0.10 (0.06, 0.16)</td>
</tr>
<tr>
<td>0.05 mg cigarettes</td>
<td>0.09 (0.05, 0.15)</td>
</tr>
<tr>
<td>Nicotine lozenge</td>
<td>0.08 (0.05, 0.12)</td>
</tr>
<tr>
<td>Total 1-HOP</td>
<td></td>
</tr>
<tr>
<td>0.3 mg cigarettes</td>
<td>0.84 (0.70, 1.02)</td>
</tr>
<tr>
<td>0.05 mg cigarettes</td>
<td>0.89 (0.71, 1.12)</td>
</tr>
<tr>
<td>Nicotine lozenge</td>
<td>0.94 (0.71, 1.24)</td>
</tr>
<tr>
<td>3-HPMA</td>
<td></td>
</tr>
<tr>
<td>0.3 mg cigarettes</td>
<td>3662 (2868, 4674)</td>
</tr>
<tr>
<td>0.05 mg cigarettes</td>
<td>3320 (2667, 4134)</td>
</tr>
<tr>
<td>Nicotine lozenge</td>
<td>3445 (2539, 4673)</td>
</tr>
<tr>
<td>S-PMA</td>
<td></td>
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<tr>
<td>0.3 mg cigarettes</td>
<td>2.21 (1.54, 3.18)</td>
</tr>
<tr>
<td>0.05 mg cigarettes</td>
<td>2.46 (1.68, 3.62)</td>
</tr>
<tr>
<td>Nicotine lozenge</td>
<td>2.69 (1.95, 3.72)</td>
</tr>
</tbody>
</table>

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-hydroxypropylmercaptopurine acid; S-PMA: S-phenylmercaptopurine acid.

1 ng/ml.
2 pmol/mg creatinine.

Addiction. Author manuscript; available in PMC 2015 September 10.
* $P < 0.05$ 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 ($P < 0.05$).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>0.3 mg nicotine cigarettes (n = 52)</th>
<th>Nicotine lozenges (n = 60)</th>
<th>0.05 mg nicotine cigarettes (n = 53)</th>
<th># abstinent</th>
<th>%</th>
<th># abstinent</th>
<th>%</th>
<th># abstinent</th>
<th>%</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Continuous abstinence</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>CO verified</td>
<td>11</td>
<td>21.2</td>
<td>21</td>
<td>35.0</td>
<td>23</td>
<td>43.4</td>
<td>0.0508</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CO and cotinine verified</td>
<td>7</td>
<td>13.5</td>
<td>11</td>
<td>18.3</td>
<td>16</td>
<td>30.2</td>
<td>0.0913</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CO verified point prevalence abstinence</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follow-up week</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>18</td>
<td>34.6</td>
<td>25</td>
<td>41.7</td>
<td>22</td>
<td>41.5</td>
<td>0.6954</td>
<td></td>
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</tr>
<tr>
<td>2</td>
<td></td>
<td>17</td>
<td>32.7</td>
<td>25</td>
<td>41.7</td>
<td>29</td>
<td>54.7</td>
<td>0.0719</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>12</td>
<td>23.1</td>
<td>22</td>
<td>36.7</td>
<td>23</td>
<td>43.4</td>
<td>0.0829</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>12</td>
<td>23.1$^a$</td>
<td>22</td>
<td>36.7$^{a,b}$</td>
<td>25</td>
<td>47.2$^b$</td>
<td>0.0357</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CO and cotinine verified point prevalence abstinence</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Follow-up week</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>12</td>
<td>23.1$^a$</td>
<td>16</td>
<td>26.7$^a$</td>
<td>24</td>
<td>45.3$^b$</td>
<td>0.0298</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>8</td>
<td>15.4$^a$</td>
<td>13</td>
<td>21.7$^a$</td>
<td>21</td>
<td>39.6$^b$</td>
<td>0.0120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>7</td>
<td>13.5$^a$</td>
<td>12</td>
<td>20.0$^{a,b}$</td>
<td>19</td>
<td>35.9$^b$</td>
<td>0.0192</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.
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.
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 (N2010=165; N2013=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.

---

1Percent 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). 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.

---

2Analyses 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.

3Analyses 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

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References

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

<table>
<thead>
<tr>
<th>Natural Log of Week 6 Cotinine</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>16</td>
<td>21</td>
<td>24</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>% in 2013 study</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>56.3</td>
<td>47.6</td>
<td>75.0</td>
<td>83.3</td>
<td>100</td>
<td>66.7</td>
<td>66.7</td>
</tr>
<tr>
<td>% smoking 0.09 mg yield</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>25.0</td>
<td>33.3</td>
<td>70.8</td>
<td>66.7</td>
<td>60.0</td>
<td>66.7</td>
<td>66.7</td>
</tr>
<tr>
<td>study CPD (Week 6)</td>
<td>0</td>
<td>2.6</td>
<td>7.9</td>
<td>8.7</td>
<td>16.9</td>
<td>17.8</td>
<td>17.2</td>
<td>11.4</td>
<td>18.0</td>
<td>18.0</td>
</tr>
<tr>
<td>non-study CPD (Week 6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0.7</td>
<td>4.2</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>% non-compliant</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>12.5</td>
<td>14.3</td>
<td>33.3</td>
<td>50.0</td>
<td>80.0</td>
<td>66.7</td>
<td>66.7</td>
</tr>
<tr>
<td>% abstinent (Week 12)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>81.3</td>
<td>57.1</td>
<td>47.1</td>
<td>16.7</td>
<td>20</td>
<td>16.7</td>
<td>16.7</td>
</tr>
</tbody>
</table>

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.
Dose–Response Effects of Spectrum Research Cigarettes

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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 deaths 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

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Reduced nicotine content cigarettes

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 inter puff 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, p = .008).
### Table 1. Study 1: Subscales of the Modified Cigarette Evaluation Questionnaire (mCEQ) by Nicotine Level

<table>
<thead>
<tr>
<th>Measurementa</th>
<th>Usual Brand</th>
<th>Lower LS</th>
<th>Intermediate LS</th>
<th>Higher LS</th>
<th>Lower vs. Intermediate Mean (SE)b</th>
<th>Lower vs. Higher Mean (SE)b</th>
<th>Intermediate vs. Higher Mean (SE)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Satisfaction</td>
<td>83.6 (1.4)</td>
<td>36.8 (4.1)</td>
<td>54.1 (4.1)</td>
<td>61.4 (4.1)</td>
<td>[t] = 3.26*, [t] = 4.65***</td>
<td>[t] = 1.38</td>
<td></td>
</tr>
<tr>
<td>Psychological Reward</td>
<td>53.7 (2.2)</td>
<td>33.5 (3.1)</td>
<td>40.9 (3.1)</td>
<td>43.6 (3.1)</td>
<td>[t] = 1.85, [t] = 2.53*</td>
<td>[t] = 0.68</td>
<td></td>
</tr>
<tr>
<td>Aversion</td>
<td>13.0 (1.7)</td>
<td>16.7 (2.8)</td>
<td>14.1 (2.8)</td>
<td>18.2 (2.8)</td>
<td>[t] = 0.73, [t] = 0.40</td>
<td>[t] = 1.14</td>
<td></td>
</tr>
<tr>
<td>Enjoyment of Sensation</td>
<td>73.1 (2.4)</td>
<td>32.7 (4.2)</td>
<td>49.3 (4.2)</td>
<td>54.8 (4.2)</td>
<td>[t] = 2.70*, [t] = 4.09**</td>
<td>[t] = 1.03</td>
<td></td>
</tr>
<tr>
<td>Craving Reduction</td>
<td>62.8 (3.3)</td>
<td>42.8 (4.5)</td>
<td>54.9 (4.5)</td>
<td>58.6 (4.5)</td>
<td>[t] = 2.07, [t] = 2.71*</td>
<td>[t] = 0.64</td>
<td></td>
</tr>
</tbody>
</table>

Notes. LS = least square; SE = standard error.

aSubscale scores were averaged across the items.
bAdjusted 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 ≤ .05. **p ≤ .01. ***p ≤ .001. ****p ≤ .0001.

*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 ± 12 vs. 74.6 ± 1.2; [t] = 3.49, p = .002; and 73.9 ± 4.5 vs. 71.3 ± 1.5, [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
Reduced nicotine content cigarettes

Table 2. Study 1: Perceived Health Risk (PHR) by Nicotine Level

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(0–100 Scale)</td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>79.8 (2.6)</td>
<td>56.6 (4.6)</td>
<td>66.7 (4.6)</td>
<td>68.3 (4.6)</td>
<td>68.3 (4.6)</td>
<td>68.3 (4.6)</td>
<td>68.3 (4.6)</td>
</tr>
<tr>
<td>Emphysema</td>
<td>77.6 (2.7)</td>
<td>60.6 (4.2)</td>
<td>67.9 (4.2)</td>
<td>70.8 (4.2)</td>
<td>70.8 (4.2)</td>
<td>70.8 (4.2)</td>
<td>70.8 (4.2)</td>
</tr>
<tr>
<td>Bronchitis</td>
<td>76.2 (3.2)</td>
<td>59.4 (4.2)</td>
<td>65.7 (4.2)</td>
<td>68.4 (4.1)</td>
<td>68.4 (4.1)</td>
<td>68.4 (4.1)</td>
<td>68.4 (4.1)</td>
</tr>
<tr>
<td>Other cancers</td>
<td>74.5 (3.0)</td>
<td>57.2 (3.9)</td>
<td>64.0 (3.9)</td>
<td>68.8 (3.9)</td>
<td>68.8 (3.9)</td>
<td>68.8 (3.9)</td>
<td>68.8 (3.9)</td>
</tr>
<tr>
<td>Heart disease</td>
<td>77.1 (3.0)</td>
<td>62.6 (3.9)</td>
<td>67.6 (3.9)</td>
<td>72.3 (3.9)</td>
<td>72.3 (3.9)</td>
<td>72.3 (3.9)</td>
<td>72.3 (3.9)</td>
</tr>
<tr>
<td>Risk of addiction</td>
<td>85.9 (2.3)</td>
<td>51.0 (4.8)</td>
<td>58.1 (4.7)</td>
<td>69.9 (4.7)</td>
<td>69.9 (4.7)</td>
<td>69.9 (4.7)</td>
<td>69.9 (4.7)</td>
</tr>
<tr>
<td>Stroke</td>
<td>70.9 (3.3)</td>
<td>58.1 (3.7)</td>
<td>62.8 (3.6)</td>
<td>68.3 (3.6)</td>
<td>68.3 (3.6)</td>
<td>68.3 (3.6)</td>
<td>68.3 (3.6)</td>
</tr>
</tbody>
</table>

Notes: LS = least square; SE = standard error.

*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 ≤ .05. **p ≤ .01. ***p ≤ .001. ****p ≤ .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,
respectively). There were no differences by gender.

Total cotinine and TNE levels among those smoking HN cigarettes had a significant decrease in CO levels (change: $22.2 \pm 16.4; |t| = 2.77, p < .003$) whereas CO did not change significantly in the IN and HN conditions compared with baseline (change: $-2.0 \pm 2.5; |t| = 0.74, p = .465$ and $-2.7 \pm 2.4; |t| = 1.12, p = .273$, 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: $-16.7 \pm 8.1; |t| = 4.15, p = .001$ 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 ($5.44 \pm 0.69$ vs. $1.68 \pm 0.72; |t| = -3.71, p = .003$ and $|t| = 2.58, p = .046$, respectively) and significantly or nearly significantly lower disliking of the cigarettes ($43.5 \pm 7.9$ vs. $91.5 \pm 8.4$ and $72.6 \pm 8.7; |t| = 4.15, p = .001$ and $|t| = 2.49, p = .058$, respectively).

**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 $|t| = 4.15, p = .001$ and $|t| = 2.49, p = .058$, respectively) and significantly or nearly significantly lower disliking of the cigarettes ($43.5 \pm 7.9$ vs. $91.5 \pm 8.4$ and $72.6 \pm 8.7; |t| = 4.15, p = .001$ and $|t| = 2.49, p = .058$, respectively).

## 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 3. Study 2: Demographics and Smoking History of All Subjects and by Nicotine Level

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total</th>
<th>Lower Nicotine</th>
<th>Intermediate Nicotine</th>
<th>Higher Nicotine</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>35</td>
<td>12 (34.3)</td>
<td>11 (31.4)</td>
<td>12 (34.3)</td>
<td>.477</td>
</tr>
<tr>
<td>Male</td>
<td>15 (42.9)</td>
<td>4 (33.3)</td>
<td>4 (36.4)</td>
<td>7 (58.3)</td>
<td>.068</td>
</tr>
<tr>
<td>Female</td>
<td>20 (57.1)</td>
<td>8 (66.7)</td>
<td>7 (63.6)</td>
<td>5 (41.7)</td>
<td>.745</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>33 (94.9)</td>
<td>11 (91.7)</td>
<td>11 (100.0)</td>
<td>11 (91.7)</td>
<td>.718</td>
</tr>
<tr>
<td>Hispanic or Latino</td>
<td>2 (5.7)</td>
<td>1 (8.3)</td>
<td>0 (0.0)</td>
<td>1 (8.3)</td>
<td>1.00</td>
</tr>
<tr>
<td>Not Hispanic/Latino</td>
<td>2 (5.7)</td>
<td>1 (8.3)</td>
<td>0 (0.0)</td>
<td>1 (8.3)</td>
<td>1.00</td>
</tr>
<tr>
<td>Race</td>
<td>1 (2.9)</td>
<td>1 (8.3)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>.585</td>
</tr>
<tr>
<td>American Indian/Alaskan native</td>
<td>1 (2.9)</td>
<td>0 (0.0)</td>
<td>1 (9.1)</td>
<td>0 (0.0)</td>
<td>.068</td>
</tr>
<tr>
<td>Asian</td>
<td>1 (2.9)</td>
<td>0 (0.0)</td>
<td>1 (9.1)</td>
<td>0 (0.0)</td>
<td>.068</td>
</tr>
<tr>
<td>Black or African American</td>
<td>7 (20.0)</td>
<td>3 (25.0)</td>
<td>1 (9.1)</td>
<td>3 (25.0)</td>
<td>.745</td>
</tr>
<tr>
<td>White</td>
<td>22 (62.9)</td>
<td>8 (66.7)</td>
<td>7 (63.6)</td>
<td>7 (58.3)</td>
<td>.745</td>
</tr>
<tr>
<td>More than one race</td>
<td>4 (11.4)</td>
<td>0 (0.0)</td>
<td>2 (18.2)</td>
<td>2 (16.7)</td>
<td>.745</td>
</tr>
<tr>
<td>Smoke menthols</td>
<td>24 (68.6)</td>
<td>8 (66.7)</td>
<td>8 (72.7)</td>
<td>8 (66.7)</td>
<td>1.00</td>
</tr>
<tr>
<td>No</td>
<td>11 (31.4)</td>
<td>4 (33.3)</td>
<td>3 (27.3)</td>
<td>4 (33.3)</td>
<td>.745</td>
</tr>
</tbody>
</table>

**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.
Reduced nicotine content cigarettes

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

### Table 4. Study 2: Levels of Urinary Cotinine and Total Nicotine Equivalents for Each Subject by Cigarette Type

<table>
<thead>
<tr>
<th>Cigarette type</th>
<th>Cotinine (nmol/ml)</th>
<th>Total nicotine equivalent (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Usual cigarettes</td>
<td>Experimental cigarettes</td>
</tr>
<tr>
<td></td>
<td>Usual cigarettes</td>
<td>Experimental cigarettes</td>
</tr>
<tr>
<td><strong>Lower nicotine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>202</td>
<td>14.0</td>
<td>4.7</td>
</tr>
<tr>
<td>204</td>
<td>6.7</td>
<td>0.1</td>
</tr>
<tr>
<td>214*</td>
<td>8.8</td>
<td>1.7</td>
</tr>
<tr>
<td>221*</td>
<td>22.6</td>
<td>5.0</td>
</tr>
<tr>
<td>231</td>
<td>41.3</td>
<td>0.5</td>
</tr>
<tr>
<td>232</td>
<td>36.3</td>
<td>17.0</td>
</tr>
<tr>
<td>234</td>
<td>16.3</td>
<td>0.1</td>
</tr>
<tr>
<td>235</td>
<td>17.7</td>
<td>8.8</td>
</tr>
<tr>
<td>238</td>
<td>59.7</td>
<td>13.7</td>
</tr>
<tr>
<td>240*</td>
<td>8.6</td>
<td>14.0</td>
</tr>
<tr>
<td>246</td>
<td>42.1</td>
<td>Missing</td>
</tr>
<tr>
<td>250*</td>
<td>14.0</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>Intermediate nicotine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>205*</td>
<td>25.3</td>
<td>2.4</td>
</tr>
<tr>
<td>210</td>
<td>25.0</td>
<td>6.6</td>
</tr>
<tr>
<td>211*</td>
<td>34.2</td>
<td>10.3</td>
</tr>
<tr>
<td>216*</td>
<td>1.3</td>
<td>1.9</td>
</tr>
<tr>
<td>225*</td>
<td>44.7</td>
<td>37.3</td>
</tr>
<tr>
<td>227</td>
<td>14.7</td>
<td>14.6</td>
</tr>
<tr>
<td>230</td>
<td>3.2</td>
<td>2.1</td>
</tr>
<tr>
<td>236</td>
<td>24.5</td>
<td>7.0</td>
</tr>
<tr>
<td>239</td>
<td>17.5</td>
<td>2.3</td>
</tr>
<tr>
<td>244</td>
<td>7.6</td>
<td>36.6</td>
</tr>
<tr>
<td>248</td>
<td>14.5</td>
<td>7.9</td>
</tr>
<tr>
<td><strong>Higher nicotine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>206</td>
<td>3.4</td>
<td>4.0</td>
</tr>
<tr>
<td>209</td>
<td>39.8</td>
<td>14.1</td>
</tr>
<tr>
<td>213</td>
<td>20.6</td>
<td>24.3</td>
</tr>
<tr>
<td>215*</td>
<td>28.7</td>
<td>38.8</td>
</tr>
<tr>
<td>222</td>
<td>7.5</td>
<td>16.3</td>
</tr>
<tr>
<td>226*</td>
<td>10.0</td>
<td>23.0</td>
</tr>
<tr>
<td>233</td>
<td>8.1</td>
<td>24.1</td>
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<td>237</td>
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<td>242</td>
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<td>245</td>
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<td>26.6</td>
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<tr>
<td>252</td>
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<td>14.0</td>
</tr>
<tr>
<td>254</td>
<td>14.5</td>
<td>20.3</td>
</tr>
</tbody>
</table>

*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.
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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

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

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Usual Brand</th>
<th>Lower LS Mean (SE)</th>
<th>Intermediate LS Mean (SE)</th>
<th>Higher LS Mean (SE)</th>
<th>Lower vs. Intermediate Mean (SE)</th>
<th>Lower vs. Higher Mean (SE)</th>
<th>Intermediate vs. Higher Mean (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotinine (nmol/ml)</td>
<td>21.8 (2.3)</td>
<td>5.0 (3.5)</td>
<td>13.4 (3.2)</td>
<td>20.3 (3.1)</td>
<td>[t] = 1.77</td>
<td>[t] = 1.55</td>
<td>[t] = 1.55</td>
</tr>
<tr>
<td>Nicotine equivalents</td>
<td>81.6 (7.8)</td>
<td>19.9 (8.1)</td>
<td>40.1 (7.6)</td>
<td>60.8 (7.1)</td>
<td>[t] = 1.81</td>
<td>[t] = 3.79*</td>
<td>[t] = 2.01</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>17.1 (1.4)</td>
<td>11.3 (2.3)</td>
<td>18.9 (2.1)</td>
<td>19.9 (1.9)</td>
<td>[t] = 2.43</td>
<td>[t] = 2.91**</td>
<td>[t] = 0.38</td>
</tr>
</tbody>
</table>

notes. ls = least square; se = standard error. excludes three subjects who smoked more than three usual-brand cigarettes during the study period. *p ≤ .01. **p ≤ .05.
Reduced nicotine content cigarettes

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 Hsukami, 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 Hsukami, Kotlyar, 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

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DECLARATION OF INTERESTS

Dorothy Hsukami 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


Electronic Cigarette Use and Progression From Experimentation to Established Smoking

Benjamin W. Chaffee, DDS, MPH, PhD, a, b Shannon Lea Watkins, PhD, a, c Stanton A. Glantz, PhD a, c, d

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.

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.
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.⁴ 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) Study¹⁴ 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 13651 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

<table>
<thead>
<tr>
<th>Outcome: smoked 100 cigarettes</th>
<th>n</th>
<th>Weighted %</th>
<th>Unadjusted</th>
<th>Adjusteda</th>
<th>Adjustedb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With Outcome</td>
<td>OR (95% CI)</td>
<td>P</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Wave 1 predictors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-cigarette never</td>
<td>646</td>
<td>9.7</td>
<td>Reference</td>
<td>—</td>
<td>Reference</td>
</tr>
<tr>
<td>E-cigarette ever</td>
<td>582</td>
<td>19.3</td>
<td>2.23 (1.55–3.21)</td>
<td>&lt;.001</td>
<td>2.07 (1.41–3.04)</td>
</tr>
<tr>
<td>E-cigarette former</td>
<td>406</td>
<td>18.6</td>
<td>2.13 (1.43–3.18)</td>
<td>&lt;.001</td>
<td>2.04 (1.33–3.12)</td>
</tr>
<tr>
<td>E-cigarette in the past 30 d</td>
<td>171</td>
<td>21.5</td>
<td>2.56 (1.58–4.14)</td>
<td>&lt;.001</td>
<td>2.22 (1.31–3.74)</td>
</tr>
<tr>
<td>Wave 1 predictors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-cigarette never</td>
<td>699</td>
<td>26.6</td>
<td>Reference</td>
<td>—</td>
<td>Reference</td>
</tr>
<tr>
<td>E-cigarette ever</td>
<td>596</td>
<td>38.8</td>
<td>1.75 (1.35–2.27)</td>
<td>&lt;.001</td>
<td>1.65 (1.26–2.15)</td>
</tr>
<tr>
<td>E-cigarette former</td>
<td>699</td>
<td>26.6</td>
<td>Reference</td>
<td>—</td>
<td>Reference</td>
</tr>
<tr>
<td>E-cigarette in the past 30 d</td>
<td>415</td>
<td>36.1</td>
<td>1.56 (1.15–2.12)</td>
<td>.004</td>
<td>1.48 (1.09–2.02)</td>
</tr>
<tr>
<td>Wave 1 predictors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-cigarette never</td>
<td>176</td>
<td>45.3</td>
<td>2.29 (1.64–3.19)</td>
<td>&lt;.001</td>
<td>2.10 (1.47–2.99)</td>
</tr>
<tr>
<td>E-cigarette ever</td>
<td>580</td>
<td>15.5</td>
<td>2.43 (1.55–3.80)</td>
<td>&lt;.001</td>
<td>2.23 (1.39–3.59)</td>
</tr>
<tr>
<td>E-cigarette former</td>
<td>406</td>
<td>18.6</td>
<td>2.41 (1.46–3.97)</td>
<td>&lt;.001</td>
<td>2.29 (1.35–3.89)</td>
</tr>
<tr>
<td>E-cigarette in the past 30 d</td>
<td>171</td>
<td>21.5</td>
<td>2.56 (1.52–4.32)</td>
<td>&lt;.001</td>
<td>2.19 (1.24–3.88)</td>
</tr>
</tbody>
</table>

Odds ratio (OR) and 95% confidence interval (95% CI) are presented for the progression from baseline cigarette smoking (100 cigarettes smoked) to established smoking (365 days of smoking history). The analyses were adjusted for sex, age, race and/or ethnicity, parent education, 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.

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 (odds ratio [OR]: 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...

bachelor degree or greater), urban residence (based on sampling units), household tobacco use (lives with ≥1 tobacco user), alcohol ever use, tobacco advertisement receptivity (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 (i.e., 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. 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. 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.
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 data13 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.
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, youth who reach higher levels of smoking are even more likely to continue to smoke. 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. 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. 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.

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. 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. 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

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The Association Between Smoking and Electronic Cigarette Use in a Cohort of Young People

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Keywords: Smoking; Electronic cigarettes; E-cigarettes; Young people; Youth; Adolescent; Longitudinal studies; Nicotine; Tobacco

ABSTRACT

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.

Keywords: Smoking; Electronic cigarettes; E-cigarettes; Young people; Youth; Adolescent; Longitudinal studies; Nicotine; Tobacco

A B S T R A C T

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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.

Keywords: Smoking; Electronic cigarettes; E-cigarettes; Young people; Youth; Adolescent; Longitudinal studies; Nicotine; Tobacco
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. © 2017 Society for Adolescent Health and Medicine. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

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 never 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
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 approval of smoking (yes, no), and public approval 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.

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).

![Figure 2. Conceptual causal diagrams for mediation and confounding.](image-url)
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.
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.
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 smoking initiation at follow-up were also assessed.
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 multi-wave 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 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.

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**Supplementary Data**

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jadohealth.2017.11.301.

**References**


In-silico study of toxicokinetics and disease association of chemicals present in smokeless tobacco products

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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, sniffed 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 in solution (pH level). 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-nitrosornornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1 butanone (NNK)) and Poly Aromatics Hydrocarbons (benzo[a]pyrene (B[a]P) (Supplementary Table 1)).

Several additives like areca nut added to SLT products to enhance their flavor, taste and aroma. Additives like areca nut contain known carcinogens and potentiate carcinogenesis (Shah et al., 2012; Sharan et al., 2012). However, the functional analysis of the compounds found...
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 TSNA and their metabolites has 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 TSNA has been studied (Adams et al., 1985, 1984; Pool-Zobel et al., 1991). The distribution and metabolism of a few TSNA has 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 TSNA, 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 in 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; Brunemann 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 pCKSM 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 pCKSM web server (Supplementary Table II).

Permeability prediction was carried out by pCKSM 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 × 10⁻⁶ cm/s. The cut off for high Caco-2 permeability in pCKSM 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 pCKSM 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
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 92 compounds 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.
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 products have been predicted as hepatotoxic as shown in Fig. 4. Fifty percent of the 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 carcinogenic/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.
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
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...
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 effect 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

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**Table 2: ADMET properties of important compounds of SLT products (For reference values, please refer Supplementary Table II).**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Class</th>
<th>Absorption</th>
<th>Solubility</th>
<th>Hepatotoxicity</th>
<th>Mutagenicity</th>
<th>Caco-2 permeability</th>
<th>CNS permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNN</td>
<td>Tobacco specific nitrosamines</td>
<td>Good</td>
<td>Good</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
</tr>
<tr>
<td>NNK</td>
<td>Good</td>
<td>Good</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>NAT</td>
<td>Good</td>
<td>Good</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>NAB</td>
<td>Good</td>
<td>Good</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>MNBA</td>
<td>N-Nitrosamino acids</td>
<td>Good</td>
<td>Optimal</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
</tr>
<tr>
<td>MNPA</td>
<td>Good</td>
<td>Optimal</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>NAzCA</td>
<td>Good</td>
<td>Optimal</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>NSAR</td>
<td>Good</td>
<td>Optimal</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Npip</td>
<td>Volatile N-nitrosamines</td>
<td>Good</td>
<td>Good</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
</tr>
<tr>
<td>NMOR</td>
<td>Good</td>
<td>Optimal</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>NDHA</td>
<td>Good</td>
<td>Too soluble</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>NDMA</td>
<td>Good</td>
<td>Optimal</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>Not predicted</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>5-Methyl chrysene</td>
<td>Poly aromatic hydrocarbons</td>
<td>Moderate</td>
<td>Very low but possible</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
</tr>
<tr>
<td>Benz[a]anthracene</td>
<td>Moderate</td>
<td>Very low but possible</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Benz[a]pyrene</td>
<td>Moderate</td>
<td>Very low but possible</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>Moderate</td>
<td>Very low but possible</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>Moderate</td>
<td>Very low but possible</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>High</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Benzo[e]pyrene</td>
<td>Moderate</td>
<td>Very low but possible</td>
<td>Toxic</td>
<td>Mutagen</td>
<td>Not predicted</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

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**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.
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 aggrivated with SLT use.

Considering the toxicokinetic behavior, 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 in of 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-availability 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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jytph.2018.03.002.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.jytph.2018.03.002.

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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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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.
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 ENDS/e-cig aerosols and pre-vaporized e-liquids in a cell free system. We also determined the source of oxidants produced from ENDS/e-cigs by a modified 2'-7',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescein derived dye to detect OX/ROS reactivity in ENDS/e-cig aerosols.
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 (H2O2) concentrations. The DCF fluorescence data are expressed as μM H2O2 equivalents referring to the concentration of the H2O2 added to the DCFH solution [16,19,24].
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% CO₂ and 3% O₂ 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...
(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% CO2 and 3% O2 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% CO2, and 3% O2 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% CO₂, and 3% O₂ 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% CO₂, and 3% O₂ controlled incubator either in a 24-well plates (2.0 cm²) or 6-well plates (9.6 cm²) 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/m³ 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 cytopsin-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 bronchoalveolar 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
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$_2$O$_2$ µM equivalents (equivalent to DCF fluorescence intensity units) as compared to air-sham group (Fig. 2A). The levels of H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ µM equivalent levels (Fig. 2B). Two of the commercially available e-liquids (Vape Dudes 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 $H_2O_2 \, \mu$M 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.

<table>
<thead>
<tr>
<th>Humectants</th>
<th>H$_2$O$_2$ equivalents (μM) $^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propylene glycol</td>
<td>120.7</td>
</tr>
<tr>
<td></td>
<td>129.0</td>
</tr>
<tr>
<td></td>
<td>127.3</td>
</tr>
<tr>
<td>Mean ± SEM $^\ddagger$</td>
<td>125.7 ± 2.5$^{***}$ (93.0) $^a$</td>
</tr>
<tr>
<td>Glycerin</td>
<td>211.5</td>
</tr>
<tr>
<td></td>
<td>305.2</td>
</tr>
<tr>
<td></td>
<td>312.4</td>
</tr>
<tr>
<td></td>
<td>360.7</td>
</tr>
<tr>
<td></td>
<td>200.2</td>
</tr>
<tr>
<td></td>
<td>145.6</td>
</tr>
<tr>
<td>Mean ± SEM $^\ddagger$</td>
<td>255.9 ± 33.6$^{***}$ (93.1) $^a$</td>
</tr>
<tr>
<td>Propylene glycol: Glycerin (50:50)</td>
<td>412.5</td>
</tr>
<tr>
<td></td>
<td>360.5</td>
</tr>
<tr>
<td></td>
<td>146.8</td>
</tr>
<tr>
<td>Mean ± SEM $^\ddagger$</td>
<td>306.6 ± 81.3$^{***}$ (97.0) $^a$</td>
</tr>
<tr>
<td>Classic tobacco (0 mg nicotine)</td>
<td>248</td>
</tr>
<tr>
<td></td>
<td>113.6</td>
</tr>
<tr>
<td></td>
<td>103.2</td>
</tr>
<tr>
<td></td>
<td>134.0</td>
</tr>
<tr>
<td></td>
<td>131.0</td>
</tr>
<tr>
<td>Mean ± SEM $^\ddagger$</td>
<td>146.0 ± 26.1$^{***}$ (81.6) $^a$</td>
</tr>
<tr>
<td>Classic tobacco (24 mg nicotine)</td>
<td>327.7</td>
</tr>
<tr>
<td></td>
<td>103.8</td>
</tr>
<tr>
<td></td>
<td>60.4</td>
</tr>
<tr>
<td>Mean ± SEM $^\ddagger$</td>
<td>164.0 ± 82.8$^{***}$ (91.5) $^a$</td>
</tr>
<tr>
<td>Pre-used heating element (without e-liquid)</td>
<td>250.5</td>
</tr>
<tr>
<td></td>
<td>192.0</td>
</tr>
<tr>
<td></td>
<td>84.2</td>
</tr>
<tr>
<td>Mean ± SEM $^\ddagger$</td>
<td>175.6 ± 48.7$^{***}$ (90.7) $^a$</td>
</tr>
<tr>
<td>Air-Sham (control)</td>
<td>3.89</td>
</tr>
<tr>
<td></td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>2.1</td>
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<td>4.9</td>
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<tr>
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<td>7.1</td>
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</table>

(Continued)
Table 2. (Continued)

<table>
<thead>
<tr>
<th>Humectants</th>
<th>H$_2$O$_2$ equivalents (μM) †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SEM ‡</td>
<td>3.2 ± 0.46</td>
</tr>
</tbody>
</table>

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$_2$O$_2$).

† Each value shown in H$_2$O$_2$ 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.

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>State of the heating element</th>
<th>New</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humectants</td>
<td>Pre-used</td>
<td>New</td>
</tr>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>15.32</td>
<td>13.06</td>
</tr>
<tr>
<td>Glycerin</td>
<td>20.65</td>
<td>34.97</td>
</tr>
<tr>
<td>Consumer refill</td>
<td>47.55</td>
<td>37.42</td>
</tr>
<tr>
<td>Air (sham)</td>
<td>1.19</td>
<td>2.08</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Clearomizer filled with e-liquid</th>
<th>Emptied clearomizer with wicked e-liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humectant</td>
<td>Trial 1</td>
</tr>
<tr>
<td>Consumer refill</td>
<td>47.55</td>
</tr>
</tbody>
</table>

Each fluorometric value shown in this table represents the H$_2$O$_2$ 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$_2$O$_2$ 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 mL 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.

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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
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

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**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.**

<table>
<thead>
<tr>
<th>Vape drops †</th>
<th>Vape dudes †</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flavors</strong></td>
<td><strong>Nicotine conc. (mg)</strong></td>
</tr>
<tr>
<td>Tobacco</td>
<td>0</td>
</tr>
<tr>
<td>Tobacco</td>
<td>6</td>
</tr>
<tr>
<td>Tobacco</td>
<td>11</td>
</tr>
<tr>
<td>Tobacco</td>
<td>18</td>
</tr>
<tr>
<td>Tobacco</td>
<td>24</td>
</tr>
<tr>
<td>Very berry</td>
<td>0</td>
</tr>
<tr>
<td>AMP</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ecto †</th>
<th>Drip †</th>
</tr>
</thead>
<tbody>
<tr>
<td>American tobacco</td>
<td>0</td>
</tr>
<tr>
<td>American tobacco</td>
<td>12</td>
</tr>
<tr>
<td>American tobacco</td>
<td>18</td>
</tr>
<tr>
<td>American tobacco</td>
<td>24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Upstate vape †</th>
<th>Encore †</th>
</tr>
</thead>
<tbody>
<tr>
<td>9x Tobacco</td>
<td>0</td>
</tr>
<tr>
<td>9x Tobacco</td>
<td>11</td>
</tr>
<tr>
<td>9x Tobacco</td>
<td>18</td>
</tr>
<tr>
<td>9x Tobacco</td>
<td>24</td>
</tr>
<tr>
<td>Marbo</td>
<td>0</td>
</tr>
<tr>
<td>Marbo</td>
<td>6</td>
</tr>
<tr>
<td>Marbo</td>
<td>11</td>
</tr>
<tr>
<td>Marbo</td>
<td>18</td>
</tr>
<tr>
<td>Marbo</td>
<td>24</td>
</tr>
<tr>
<td>Mountain dew</td>
<td>18</td>
</tr>
</tbody>
</table>

† 167 μl e-liquid added to final volume of 5 ml DCFH solution, equivalent to volume of H2O2 added to attain a fluorometric value of approximately 50 FIU. DCF fluorescence for 50 μM H2O2 standard (50.02), humectant polyethylene glycol (0.43), humectant glycerin (1.3) and vehicle/water (0.03).

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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 nic-
otine; 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, lead-
ing 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 differ-
ent than control, similarly to cell treatments with 2.5% e-liquid/humectant (CSE; 89.4 ± 5.86% viabi-
lity in means ± SD). Therefore, the lung fibroblasts were more sensitive to CSE than e-liq-
uids/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 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
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
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).

### Table 5. Effect of e-liquids on HFL-1 cell viability in small 24-well culture area after 24 hours.

<table>
<thead>
<tr>
<th>E-liquid (%)</th>
<th>Nicotine concentration (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>UV</td>
<td>E</td>
</tr>
<tr>
<td>0.5</td>
<td>85.9</td>
</tr>
<tr>
<td>5.0</td>
<td>42.6</td>
</tr>
<tr>
<td>10.0</td>
<td>20.7</td>
</tr>
</tbody>
</table>

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.

*T values below 50% viability in 0.5% e-Liquid.
* NS compared to 0 mg nicotine.

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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.
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 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 Induces Oxidative Stress and Inflammation

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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.
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 GSSG) levels of glutathione 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 suggest 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 detectable 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 sidestream/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


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.

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...
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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The author declares that they have no competing interests.

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Multigenerational and transgenerational inheritance of drug exposure: The effects of alcohol, opiates, cocaine, marijuana, and nicotine

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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.

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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, 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 “germ-line-mediated inheritance of epigenetic information 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).

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 Lynamsey, 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<sub>A</sub> 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<sub>A</sub> 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 (Gоворок и 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 regulates long-term repeat (LTR) transposons through a decrease in methylation (Pononarev et al., 2012). LTR transposons are transposable elements (TEs) that are typically silenced by the presence of DNA methylation.
Upregulated in the cortex of alcoholics (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 hypomethylation 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

### Table 1

Multigenerational and Transgenerational phenotypes of F0 drug exposure.

<table>
<thead>
<tr>
<th>Drug</th>
<th>F0 exposure</th>
<th>Generation affected</th>
<th>Sex of offspring affected</th>
<th>Phenotype</th>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Behavior</td>
<td>Alcohol</td>
<td>Paternal F1</td>
<td>Both</td>
<td>Increased sensitivity to amphetamine</td>
<td>Rat</td>
<td>Abel, 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paternal F1</td>
<td>Male</td>
<td>Reduced alcohol intake and increased behavioral response to alcohol</td>
<td>Mouse</td>
<td>Finegersh and Homanics, 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paternal F1</td>
<td>Both</td>
<td>Decreased fear and increased aggression</td>
<td>Mouse</td>
<td>Meek et al., 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paternal F1</td>
<td>Male</td>
<td>Decreased attention and increased impulsivity</td>
<td>Mouse</td>
<td>Kim et al., 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paternal F1</td>
<td>Male</td>
<td>Enhanced passive avoidance learning and deficits in T-maze</td>
<td>Mouse</td>
<td>Abel and Lee, 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paternal F1</td>
<td>Female</td>
<td>Deficits in passive avoidance learning</td>
<td>Rat</td>
<td>Abel and Tan, 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paternal F1</td>
<td>Both</td>
<td>Deficits in radial arm maze learning</td>
<td>Rat</td>
<td>Wozniak et al., 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paternal F1</td>
<td>Male</td>
<td>Hyperactivity attenuated by physostigmine treatment</td>
<td>Rat</td>
<td>Abel, 1994</td>
</tr>
<tr>
<td></td>
<td>Morphine</td>
<td>Maternal F1</td>
<td>Both</td>
<td>Increased morphine sensitization (locomotor)</td>
<td>Rat</td>
<td>Byrnes, 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maternal F1</td>
<td>Male</td>
<td>Decreased locomotor response to D1/D2 agonist</td>
<td>Rat</td>
<td>Byrnes et al., 2013</td>
</tr>
<tr>
<td></td>
<td>Cocaine</td>
<td>Paternal F1</td>
<td>Male</td>
<td>Resilience to cocaine self-administration</td>
<td>Rat</td>
<td>Vassoler et al., 2013b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maternal F1</td>
<td>Male</td>
<td>Increased sensitivity to cocaine</td>
<td>Rat</td>
<td>Sasaki et al., 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paternal F1</td>
<td>Female</td>
<td>Learning deficits</td>
<td>Mouse</td>
<td>He et al., 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paternal F1</td>
<td>Both</td>
<td>Increase in depression-like phenotype, no change in anxiety or learning and memory</td>
<td>Mouse</td>
<td>Killinger et al., 2012</td>
</tr>
<tr>
<td></td>
<td>CB1/CB2 agonist (WIN 55, 212-12)</td>
<td>Maternal F1</td>
<td>Male</td>
<td>Increased morphine CPP</td>
<td>Rat</td>
<td>Byrnes et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Δ9-THC</td>
<td>Both parents exposed and mated together</td>
<td>Female</td>
<td>Both</td>
<td>Increased morphine sensitization (locomotor)</td>
<td>Rat</td>
</tr>
<tr>
<td></td>
<td>Nicotine</td>
<td>Maternal F1</td>
<td>F1, F2, F3</td>
<td>Hyperactive, decreased attention</td>
<td>Mouse</td>
<td>Zhu et al., 2014</td>
</tr>
<tr>
<td>Neurochemical</td>
<td>Alcohol</td>
<td>Paternal F1</td>
<td>Both</td>
<td>Decreased DAT in cortex and striatum</td>
<td>Mouse</td>
<td>Kim et al., 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paternal F1</td>
<td>Male</td>
<td>Decreased beta-endorphin in hypothalamus</td>
<td>Rat</td>
<td>Cicero et al., 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maternal F1, F2, F3</td>
<td>Both</td>
<td>Decreased POMC expression in arcuate nucleus</td>
<td>Rat</td>
<td>Govorko et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Morphine</td>
<td>Paternal F1</td>
<td>Female</td>
<td>Increased beta-endorphin in hypothalamus</td>
<td>Rat</td>
<td>Cicero et al., 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maternal F1</td>
<td>Male</td>
<td>Increased kappa-opioid receptor in response to D1/D2 agonist in nucleus accumbens</td>
<td>Rat</td>
<td>Byrnes et al., 2013</td>
</tr>
<tr>
<td></td>
<td>Cocaine</td>
<td>Maternal F1, F2</td>
<td>Male</td>
<td>Increased D2 receptor in response to D1/D2 agonist in nucleus accumbens</td>
<td>Rat</td>
<td>Byrnes et al., 2013</td>
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<tr>
<td></td>
<td></td>
<td>Maternal F1</td>
<td>Male</td>
<td>Increased quinpirole induced corticosterone</td>
<td>Rat</td>
<td>Byrnes et al., 2013</td>
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<tr>
<td></td>
<td>Δ9-THC</td>
<td>Maternal F1</td>
<td>Female</td>
<td>Increased Opr1 in nucleus accumbens</td>
<td>Rat</td>
<td>Vassoler et al., 2013a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Both parents exposed and mated together</td>
<td>F1</td>
<td>Both</td>
<td>Decreased expression of cannabinoid, dopamine, and glutamate receptors, reduced NMDA binding and enhanced LTD in the dorsal striatum</td>
<td>Rat</td>
</tr>
<tr>
<td>Structural</td>
<td>Alcohol</td>
<td>Paternal F1</td>
<td>Both</td>
<td>Cortical thickening</td>
<td>Rat</td>
<td>Jamerson et al., 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paternal F1</td>
<td>Both</td>
<td>No change in pyramidal cells of hippocampus</td>
<td>Rat</td>
<td>Zajac et al., 1989</td>
</tr>
<tr>
<td></td>
<td>Morphine</td>
<td>Paternal F1</td>
<td>Both</td>
<td>Decreased hippocampal LTP</td>
<td>Rat</td>
<td>Sarkaki et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paternal F1</td>
<td>Both</td>
<td>Decreased hippocampal LTD</td>
<td>Rat</td>
<td>Sarkaki et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paternal F2</td>
<td>Both</td>
<td>Decreased synaptophysin</td>
<td>Rat</td>
<td>Vyssotski et al., 2011</td>
</tr>
</tbody>
</table>
methylation at CpGs in H19 in sperm and F2 male offspring with deceased methylation at H19 in brains (Stouder et al., 2011). Hypermethylation 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 arcuate nucleus 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; parthenogenetic activation occurs in oocytes bathed in alcohol through intracellular oscillations of free calcium ions (Cutbertson, 1983). Thus, in female animals alcohol use affects puberty, disrupts menstrual cycling, and alters reproductive function (Enamuelu 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-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 corticosterone 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 corticosterone (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 multigenational 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 (H3Ac), 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 H3Ac 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 Gα-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 51–58% 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) Val108/108Met 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, 1998). 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 administrations 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 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 a CB1 agonist produce F1 females offspring with increased Opnm1 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 (Furst et al., 2013; Porcella et al., 1998). Cannabinoid 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 PISK/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 likelihood 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 (Hillemecher 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. Gamaetes 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 δ4) 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
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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.
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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, 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 worldwide. 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 Bio-medical 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 x 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 x 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](http://www.biomedcentral.com/1741-7015/10/129)

**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.
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 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, 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% O₂-5% CO₂ (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
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.

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.
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 $R_{rs}$, and a decrease in total airway $C_{dyn}$ of the respiratory system, not only in F1 rats [20], but also in F2 rats ($P < 0.01$ versus control for both $R_{rs}$ and $C_{dyn}$), 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 $R_{rs}$ and decrease in $C_{dyn}$ in F2 rats ($P < 0.01$ versus nicotine for both $R_{rs}$ and $C_{dyn}$) (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 $R_{rs}$ and a decrease in $C_{dyn}$ in both the males and females of the F1 and F2 generations ($P < 0.05$, males versus females, for both $R_{rs}$ and $C_{dyn}$).

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
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

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**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.
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 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.
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 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.](image-url)
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 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.
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, which would be unlikely to be effectively targeted interventions to modulate the 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 teratogenic 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 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.
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 germine 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; Rs: 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.

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Perinatal nicotine-induced transgenerational asthma

Virender K. Rehan, Jie Liu, Reiko Sakurai, and John S. Torday
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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 intruterine exposure. We hypothesized that asthma transmission to offspring following perinatal nicotine exposure is not restricted up to F2 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 F0 gestating dams would transmit asthma transgenerationally to F3 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 F3 offspring following perinatal nicotine exposure of F0 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, 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 F0 pregnancy (17, 35), asthma can be inherited by rat offspring up to the F2 generation, i.e., multigenerationally. Now, we hypothesize that asthma transmission to offspring following perinatal nicotine exposure is not restricted up to F2 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 F0 gestating dams would transmit asthma transgenerationally to F3 offspring.

 methodologies

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 (F0) 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 F1 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 F1 male and female littermates were weaned at PND21 and maintained in separate cages as breeders to generate F2 rats, but without any subsequent exposure to nicotine in the F1 or F2 pregnancies. F1 pups were similarly obtained, using F2 offspring as breeders. At PND21, F3 pups underwent studies similar to those performed on F1 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 (Rs) 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 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 4.1 HEPES, and 5.5 glucose) at 16°C. The tracheal rings were then placed in 1 ml Tyrode's solution, which was maintained at 16°C and gassed with 95% O2-5% CO2. The rings were allowed to equilibrate for 30 min and placed on an isometric transducer (model 1240;transtek, Decatur, IL). Each tracheal ring was stretched to a force of 0.1 mN and suspended between two stainless steel clamps. Following equilibration, the rings were pretreated with 1 μmol/l of norepinephrine for 5 min. Next, the rings 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 (Rs) and dynamic compliance (Cdyn) were subsequently measured and plotted as a function of the methacholine concentration administered.

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25.0 NaHCO₃, 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% O₂-5% CO₂ (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 stretched to its optimal resting tension, which was achieved by measurement of isometric force, as described previously (5).

Fig. 1. Effect of perinatal nicotine exposure on total respiratory system resistance and compliance in F₁ and F₃ 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 F₁ (left) and F₃ (right) rat offspring of mixed gender, even though the F₁ rats were not exposed to any nicotine during the F₁ and F₂ gestations. Values are means ± SE; n = 10–12 rats for each group. *P < 0.05 and **P < 0.01 vs. control.

For tracheal tension measurements, each tracheal ring was initially stretched to its optimal resting tension, which was achieved by 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 measurement of isometric force, as described previously (5).

Fig. 2. Effect of perinatal nicotine exposure on total respiratory system resistance and compliance in F₁ and F₃ 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 F₁ (left) and F₃ (right) male (open diamonds) and female (closed diamonds) rats even though the F₁ and F₂ 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.

(A × 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 F₁ and F₃ 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-mm³ 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 1 (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 9-in. Pasteur pipette. The tissue was

Fig. 2. Effect of perinatal nicotine exposure on total respiratory system resistance and compliance in F₁ and F₃ 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 F₁ (left) and F₃ (right) male (open diamonds) and female (closed diamonds) rats even though the F₁ and F₂ 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.
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 × 10⁸ cells, as determined with a Coulter particle counter (Beckman-Coulter, Hialeah, FL). The cell suspension was then added to tissue culture flasks (75 cm²) 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 F₁ and F₃ generation pups was performed according to previously described methods (37). The protein concentration of the supernatant was measured by the Bradford method, quantitated by light absorbance using a Nanodrop spectrophotometer (NanoDrop Instruments, Wilmington, DE). The structural integrity of the RNA was assessed by double-immunofluorescence staining. Briefly, 5-μm sections were incubated with a mouse monoclonal antibody against fibronectin (1:50 dilution, catalog no. SC-7196; Santa Cruz Biotechnology, Santa Cruz, 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>
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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 Pro-Long 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 F3 generation rats at PND21 following a methacholine challenge and compared these data with our previously published data in F1 generation rats [please note that F1 generation data included here for comparison with F3 generation data have been published previously (35)]. Similar to the F1 generation data, compared with the control group, with perinatal nicotine exposure only to F0 dams, there was a significant increase in Rrs and a decrease in Cdyn of the total respiratory system following the methacholine challenge in F3 rats, even though the F3 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 F0 dams, the total airway Rrs increased, and the total airway Cdyn decreased in both the males and females in both the F1 and F3 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 F1 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 F3 generation and found a similar differential gender effect in the F3 generation as well (Fig. 3). It is important to point out that the gender composition and male-to-female ratios of the F1 and F3 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 F3 generation rats. Similar to our findings for the changes in the levels of these markers in F1 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 F3 rats (Fig. 4, A and B), providing further evidence for the nicotine-induced TG transmission of the lung cellular/molecular phenotype from the F1 through the F3 generation. The upregulation of fibronectin and downregulation of PPARγ protein levels in both F1 and F3 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 F1 and F2, 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., F1–F3. As in the previous study, we have observed significant effects of nicotine treatment on lung function in the F3 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 F1 and F2 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 F3 generation, we predict that its upregulation will normalize the asthma-like phenotype here too. Furthermore, in line with our previous data in F1 offspring, there was a gender-specific tracheal contractility response in F3 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 F1 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 weights (46–50). Similar to the F1 generation, in our study of the F3 generation, the observation of a TG effect of perinatal nicotine exposure on the levels of mesenchymal markers of airway differentiation in lung fibroblasts derived from F1 and F3 rats. Compared with controls, with nicotine exposure to F0 dams, mRNA (A) and protein (B) levels of fibronectin increased, whereas that of peroxisome proliferator-activated receptor γ (PPARγ) decreased in fibroblasts isolated from F1 and F3 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 F1 and F3 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; $n = 3$. AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00078.2013 • www.ajplung.org

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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 F3 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 F0 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).

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Paternal nicotine exposure defines different behavior in subsequent generation via hyper-methylation of mmu-miR-15b

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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 worldwide1 and it has recently been associated with mental disorders, such as depression, attention-deficit 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 disorder3, 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 addiction5. Most mental disorders, including MDD and ADHD, are considered complex familial aggregative or heritable traits6. 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 progeny7–10. ADHD is a developmental neuropsychiatric disorder, 11–16. ADHD is a developmental neuropsychiatric disorder,
whose pathogenesis may be influenced by multiple environmental factors, including smoking and alcohol, and chemicals such as lead and polychlorinated biphenyls17. A limited number of studies have reported that paternal smoking influences the health of the next generation14, but the effects on the mental health of offspring 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 depression15; this effect might be attributable to small non-coding RNAs (sncRNAs) in sperm, which can transmit acquired traits to progeny20. Other epigenetic modifications, such as DNA methylation and histone modification, could also contribute to the passing of non-genetic changes to future generations21–23. These findings provide potential mechanisms for the intergenerational effects of paternal smoking or nicotine exposure. However, despite these advances, the current understanding of the father-to-offspring 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. Through 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 group (F0-smo) exhibited significantly longer immobility times (Fig. 1A, P = 0.001) in the forced swim test and reduced sucrose preference (Fig. 1B, P = 0.028) compared with the non-smoking controls (F0-nos). In the open field test, F0-smo mice moved significantly 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 field tests (Fig. 1G–J), no significant differences between the two groups were detected.

The F1-nos and F1-smo offspring were obtained, and subsequently, animal behavioral tests were conducted. In the forced swim test, the total time of immobility was significantly 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 field 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 significantly 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 significantly different between the two groups. No significant 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 alternations 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 significantly 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–I), no significant 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 significantly shortened in the paternal nicotine-treated group compared with the controls (Fig. 2K, P = 0.001). In the open field 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 significantly elevated compared with the F1-con group, while other parameters (Fig. 2S,T) were not significantly different. Additionally, no significant 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 offspring. 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 confirmed 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...
Gsk3 were all significantly up-regulated in nicotine-treated mice (Fig. 3C). In a previous study, 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 significantly 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. 3C) 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 significantly down-regulated, while phosphorylation (p-GSK3α/β) was elevated after
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 significantly 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 offspring 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 significant differences were observed between the controls and the nicotine-treated samples (SFig. 3B and C). As
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 significantly 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. Specifically, nicotine treatment significantly 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 significantly 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 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 lqRPKM 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 \((n = 3)\).
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 reflecting 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.
Nicotine induced DNA hyper-methylation in the CpG island shore region of \textit{mmu-miR-15b} in F1 mouse brains and F0 mouse spermatozoa. To further examine whether an epigenetic element of \textit{mmu-miR-15b} is involved in paternal imprinting, we performed DNA methylation analyses of the promoter region of the \textit{mmu-miR-15b} gene. The promoter region of \textit{mmu-miR-15b} lacks CGIs; however, the enrichment of CpG sites in the CGI shore region (Fig. 6A) indicated that \textit{mmu-miR-15b} might be an epigenetically modifiable 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 \textit{mmu-miR-15b} in the CGI shore region were significantly 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 verification test was conducted using TM3 cells and the results confirmed that \textit{mmu-miR-15b} expression is elevated by 5′-aza-dC, which attenuated the methylation levels in the CGI shore region of \textit{mmu-miR-15b} (Fig. 6F and G).

\textbf{Overexpression of mmu-miR-15b recapitulates a depression-like phenotype.} Furthermore, we assessed the neurobehavioral effects of mmu-miR-15b and WNT4 \textit{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 \textit{Wnt4} gene transfer in the murine thalamus are shown in Fig. 7A and B, and those for
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 efficiency 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-field 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 significantly 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 significant 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-field 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 significantly 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 significantly 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 significant 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 significant 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 systems25. However, maternally provided social inheritance is unlikely in our paternal nicotine exposure model 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 disorders26. Recent studies have suggested important roles for the Wnt signaling pathway in bipolar disorder (BP)27 and MDD28. 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 arborization29,30. Moreover, the expression level of GSK3β has been associated with anxiety-like and depression-like behaviors29,30. 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 neurons31,32. Deficiencies in the signaling pathways that normally maintain the inhibition of GSK3 could induce the up-regulation of GSK3 activity, thereby promoting susceptibility to depression32. 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 disorders34. The results of the present study indicated that the over-expression of mmu-miR-15b in the mouse brain induced hyperactivity 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 schizophrenia35 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 offpring37. 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
epigenetic mechanisms. The expression level of mmu-miR-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, but the precise mechanism remains unknown. We proposed that the alterations of the ncRNA levels in sperm might only be markers or side effects of other epigenetic modifications. 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. Such epigenetic modifications are subsequently passed to the next generation and imprint the brain, leading to the induction of neurobiological abnormalities. Moreover, the intergenerational effects mediated by mmu-miR-15b were not passed down to the F2 generation. This phenomenon might suggest the reprogramming of DNA methylation during spermatogenesis in F1-nic mice. Evolutionarily, the reprogramming of epigenetic modifications can be considered as a protective mechanism that insulates further generations from the intergenerational effects of environmental 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 modifications may mediate the intergenerational inheritance of nicotine-induced neuropsychological disorders. 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 offspring.

**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/6J 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 (>20 cigarettes/day)\(^9\). The mice received nicotine treatment 4 times per day (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/6J mice to generate the F1 offpring, 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 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 × 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 offpring (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 × 27.5 cm × 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 × 5 cm × 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 × 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 first 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 ± 1 °C. The time taken to reach the fl atting state (i.e., an immobile state with only small limb movements to remain afloat) 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 purified 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 purified RNA submitted for sequencing. Following reverse transcription with random primers, the samples were segmented, and the adaptors were added. A TruSeq™ 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 “Cufflinks”. 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 50IU/ml penicillin, 50IU/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 final concentrations of 0, 0.5, 1 and 2μM. 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.** Purified 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; Mailbio) 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. 21185), 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, 9272S), 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 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 internal reference gene U6. The relative quantification (RQs) were performed using the \(2^{-\Delta\Delta Ct} \) method, where RQ or the fold-change is equal to \(2^{-\Delta\Delta Ct} \)

**Immunofluorescence (IF).** The PFA-fi ed brain tissue of the mice from the two groups was immersed in 4% paraformaldehyde in PBS at room temperature for 1 h. After washing with PBS, the sections were blocked with 5% BSA (in TBS, pH 7.4) for 30 min, followed by rinsing with PBS (3 × 5 min) and overnight incubation with the primary antibodies at 4 °C. After washing with PBS, the sections were incubated with the indicated FITC-conjugated mouse anti-goat IgG (Proteintech, SA00003-2, 1:200 in TBS) at 37 °C for 1 h. After washing three times with PBS, 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).** Purified genomic DNA was isolated using the phenol-chloroform extraction method. Next, 1 μg of purified DNA was treated using the Methylation DNA Modification Kit (Epigentek) according to the manufacturer’s instructions. The eluted DNA (5 μl) was PCR amplified using mmu-miR-15b-specific bisulfite sequencing primers (15b-BSP-S: 5'-GAAGTTTGTGGAGATTTTTTGAG-3'; and 15b-BSP-A: 5'-AAAAAACAATCCCAACATAAAAAT-3'). The PCR product (303 bp) was recovered, purified 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.mpimp.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 amplified with two sets of MSP primers (15b-M-S: 5'-AGGAATTTTATGTAGTTTTTACCG -3' ; and 15b-M-A: 5'-ACACCTACACCTGATAATACCCG-3'; 15b-U-S:...
and the connective tissue was removed with 10% H₂O₂. Burr holes were drilled a

**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% H₂O₂. 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. Significant differences are indicated by “*” (P < 0.05), and extremely significant 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**


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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.

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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).12–14 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.15–18 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/mL) 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
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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”26,29) 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, avoidance, 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 5.83 \(SD = 1.60, range = 2–9\). 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 \(t(67) = -4.635, p < .001\), heart disease \(t(67) = -3.953, p < .001\), emphysema \(t(67) = -4.521, p < .001\), ...
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.

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, 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 research20,24 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...
use of non-study (ie, normal nicotine) and VLNC cigarettes, abstinence rates decreased. 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. 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 (e.g., 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. 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.

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### Declaration of Interests

None declared.

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### References

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’-hydroxycotinine/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.
(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]. 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

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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 \textit{CYP2A6} decrease- or loss-of-function alleles) to avoid the confound of higher or lower frequencies of \textit{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).

\textbf{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 \textit{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 \textit{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 \textit{CYP2A6} genotype and BMI (Table 2b and c). These models indicated that, of the variables studied in both tribal populations, \textit{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 \textit{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 \textit{CYP2A6*1B} genotype and tribal population were included as predictor variables. Both variables independently accounted for >6% of the variation in NMR (\textit{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 \textit{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 \textit{CYP2A6*1B} genotype and NMR is similar in both tribal populations.

\textbf{Associations between smoking behaviors and \textit{CYP2A6} genotype and activity}

Relationships between \textit{CYP2A6} genotype and NMR and smoking behaviors were assessed. There was no difference in the proportion of current and former smokers who were \textit{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 \textit{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.

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**References**


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

<table>
<thead>
<tr>
<th>CYP2A6 Allele</th>
<th>Activity</th>
<th>Allele Frequency (%)</th>
<th>Northern Plains</th>
<th>Southwest</th>
<th>(P) value (^b)</th>
<th>Alaska Native</th>
<th>Caucasian</th>
<th>African American</th>
<th>Japanese</th>
<th>Chinese</th>
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<td>*1B</td>
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<td>65.3</td>
<td>26.7–35.0</td>
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<td>0.3</td>
<td>0.6</td>
<td>0.53</td>
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<td>1.1–5.3</td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td>*4</td>
<td>Inactive</td>
<td>1.6</td>
<td>0.3</td>
<td>0.07</td>
<td>14.5</td>
<td>0.13–4.2</td>
<td>0.5–2.7</td>
<td>17.0–24.2</td>
<td>4.9–15.1</td>
<td>10.8–11.0</td>
<td></td>
</tr>
<tr>
<td>*7</td>
<td>Inactive</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>0–0.3</td>
<td>0</td>
<td>6.3–12.6</td>
<td>2.2–9.8</td>
<td>3.6–9.8</td>
<td></td>
</tr>
<tr>
<td>*9</td>
<td>Decrease</td>
<td>11.9</td>
<td>20.9</td>
<td>0.0002</td>
<td>8.9</td>
<td>5.2–8.0</td>
<td>5.7–9.6</td>
<td>19.0–20.7</td>
<td>15.6–15.7</td>
<td>19.6–22.3</td>
<td></td>
</tr>
<tr>
<td>*12</td>
<td>Decrease</td>
<td>0.3</td>
<td>0.3</td>
<td>0.95</td>
<td>0.4</td>
<td>0–3.0</td>
<td>0–0.4</td>
<td>0–0.8</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>*17</td>
<td>Inactive</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>7.1–10.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>*35</td>
<td>Decrease</td>
<td>0</td>
<td>0.3</td>
<td>0.17</td>
<td>0</td>
<td>0</td>
<td>2.5–2.9</td>
<td>0.8</td>
<td>0.5</td>
<td></td>
<td>–</td>
</tr>
</tbody>
</table>

\(^a\)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

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>95% CI for B</th>
<th>Beta</th>
<th>Variation in NMR accounted for by each variable</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a. Both Tribal Populations Combined</strong> (n=150 included in model)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A6 Genotype</td>
<td>0.18</td>
<td>0.11 to 0.25</td>
<td>0.38</td>
<td>14.52%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender (male=1, female=0)</td>
<td>−0.04</td>
<td>−0.14 to 0.05</td>
<td>−0.07</td>
<td>0.45%</td>
<td>0.37</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.0001</td>
<td>−0.003 to 0.004</td>
<td>0.02</td>
<td>0.03%</td>
<td>0.82</td>
</tr>
<tr>
<td>Traditional Tobacco user (yes=1, no=0)</td>
<td>−0.01</td>
<td>−0.12 to 0.10</td>
<td>−0.02</td>
<td>0.03%</td>
<td>0.81</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>−0.008</td>
<td>−0.01 to 0.001</td>
<td>−0.17</td>
<td>1.35%</td>
<td>0.03</td>
</tr>
<tr>
<td>CPD</td>
<td>0.003</td>
<td>−0.004 to 0.01</td>
<td>0.07</td>
<td>0.45%</td>
<td>0.36</td>
</tr>
<tr>
<td>Tribal population (NP=1, SW=2)</td>
<td>−0.10</td>
<td>−0.20 to 0.002</td>
<td>−0.15</td>
<td>2.05%</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>b. Northern Plains Smokers</strong> (n=108 included in model)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A6 Genotype</td>
<td>0.21</td>
<td>0.13 to 0.30</td>
<td>0.45</td>
<td>19.71%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>−0.007</td>
<td>−0.02 to 0.001</td>
<td>−0.16</td>
<td>2.40%</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>c. Southwestern Smokers</strong> (n=48 included in model)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A6 Genotype</td>
<td>0.11</td>
<td>0.0001 to 0.22</td>
<td>0.29</td>
<td>8.12%</td>
<td>0.05</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>−0.005</td>
<td>−0.02 to 0.005</td>
<td>−0.14</td>
<td>1.99%</td>
<td>0.33</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>95% CI for B</th>
<th>Beta</th>
<th>Variation in NMR accounted for by each variable (^a)</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a. Original Model (n=97)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A6*1B Genotype (^b)</td>
<td>0.14</td>
<td>0.04 to 0.23</td>
<td>0.27</td>
<td>7.34%</td>
<td>0.006</td>
</tr>
<tr>
<td>Tribal Population (NP=1, SW=2)</td>
<td>–0.17</td>
<td>–0.30 to 0.04</td>
<td>–0.25</td>
<td>6.00%</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>b. Original Model Plus Interaction Term “Genotype x Tribe” (n=97)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2A6*1B Genotype (^b)</td>
<td>0.09</td>
<td>–0.20 to 0.37</td>
<td>0.17</td>
<td>0.32%</td>
<td>0.55</td>
</tr>
<tr>
<td>Tribal Population (NP=1, SW=2)</td>
<td>–0.23</td>
<td>–0.56 to 0.11</td>
<td>–0.33</td>
<td>1.64%</td>
<td>0.19</td>
</tr>
<tr>
<td>Genotype x Tribe Interaction (^c)</td>
<td>0.04</td>
<td>–0.16 to 0.23</td>
<td>0.13</td>
<td>0.12%</td>
<td>0.72</td>
</tr>
</tbody>
</table>

\(^a\) 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).
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-tetraddeuterocotinine). 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:

\[
CL_{\text{NIC}} = \frac{[\text{Dose}_{\text{NIC-d2}}]}{[\text{AUC}_{\text{NIC-d2}}]}
\]

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:

\[
f = \frac{[\text{AUC}_{\text{COT-d2}}]/[\text{Dose}_{\text{NIC-d2}}]}{[\text{CL}_{\text{NIC-d2}}]} 
\times [\text{CL}_{\text{COT-d4}}].
\]

The metabolic clearance of nicotine by way of the cotinine pathway was computed as \([CL_{\text{NIC-d2}} \times f]\). 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 \(CL_{\text{COT}}/f\) is the factor (K) that converts plasma cotinine concentration to daily intake of nicotine derived from tobacco use as follows: \(D_{\text{NIC}} = [P_{\text{COT}}] \times [CL_{\text{COT}}/f] = P_{\text{COT}} \times K\), where \(D_{\text{NIC}}\) is the daily intake of nicotine from smoking (mg/24 hr) and \(P_{\text{COT}}\) 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 \(*I/*I (62\%\) followed by \(*I/*17 (14\%), *I/*35 (7\%) and *I/*9 and *I/*4H (3.7\% each).\)

**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, \( \text{CL}_{\text{COT}}/\text{f} \), 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 *26 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.

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References


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**

<table>
<thead>
<tr>
<th>CYP2A6 Genotype activity</th>
<th>n</th>
<th>Plasma 3HC/COT baseline</th>
<th>Plasma 3HCd2/COTd2</th>
<th>CLNIC (mL · min⁻¹ · kg⁻¹)</th>
<th>CLNIC-COT (mL · min⁻¹ · kg⁻¹)</th>
<th>Total CLCOT (mL · min⁻¹ · kg⁻¹)</th>
<th>NIC t½ (min)</th>
<th>COT t½ (min)</th>
<th>Total CLCOT/f</th>
<th>Baseline COT (ng/mL)</th>
<th>Estimated NIC intake (mg/24h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1/*1</td>
<td>37</td>
<td>0.45 (0.27)</td>
<td>0.19 (0.12)</td>
<td>17.6 (4.4)</td>
<td>12.4 (4.2)</td>
<td>0.57 (0.23)</td>
<td>124 (31)</td>
<td>977 (222)</td>
<td>0.092 (0.033)</td>
<td>223.4 (130.6)</td>
<td>20.3 (13.3)</td>
</tr>
<tr>
<td>*1/*17</td>
<td>8</td>
<td>0.29 (0.10)</td>
<td>0.12 (0.05)</td>
<td>16.3 (3.6)</td>
<td>10.7 (2.8)</td>
<td>0.42 (0.17)</td>
<td>134 (33)</td>
<td>1107 (131)</td>
<td>0.074 (0.016)</td>
<td>205.7 (60.9)</td>
<td>15.1 (3.4)</td>
</tr>
<tr>
<td>*1/*9</td>
<td>2</td>
<td>0.35 (0.21)</td>
<td>0.16</td>
<td>21.6 (4.6)</td>
<td>14.8 (2.3)</td>
<td>0.52 (0.01)</td>
<td>97 (12)</td>
<td>868 (119)</td>
<td>0.079</td>
<td>153.2 (27.5)</td>
<td>11.9 (0.5)</td>
</tr>
<tr>
<td>*1/*24</td>
<td>1</td>
<td>0.54</td>
<td>0.15</td>
<td>16.3</td>
<td>13.9</td>
<td>0.39</td>
<td>147</td>
<td>960</td>
<td>0.065</td>
<td>258.7</td>
<td>16.8</td>
</tr>
<tr>
<td>*1/*41</td>
<td>2</td>
<td>0.22 (0.02)</td>
<td>0.14 (0.03)</td>
<td>13.8 (0.7)</td>
<td>8.8 (2.6)</td>
<td>0.45 (0.02)</td>
<td>152 (7)</td>
<td>1197 (128)</td>
<td>0.085 (0.008)</td>
<td>227.7 (22.8)</td>
<td>19.2 (0.2)</td>
</tr>
<tr>
<td>*1/*27</td>
<td>1</td>
<td>0.31</td>
<td>0.13</td>
<td>19.5</td>
<td>15.2</td>
<td>0.39</td>
<td>108</td>
<td>1092</td>
<td>0.067</td>
<td>67.3</td>
<td>4.4</td>
</tr>
<tr>
<td>*1/*2</td>
<td>1</td>
<td>0.25</td>
<td>0.13</td>
<td>14.6</td>
<td>11.6</td>
<td>0.41</td>
<td>151</td>
<td>1125</td>
<td>0.062</td>
<td>320.3</td>
<td>19.9</td>
</tr>
<tr>
<td>*1/*35</td>
<td>3</td>
<td>0.24 (0.06)</td>
<td>0.10 (0.06)</td>
<td>11.6 (5.5)</td>
<td>7.8 (3.1)</td>
<td>0.32 (0.11)</td>
<td>179 (35)</td>
<td>1387 (355)</td>
<td>0.048 (0.011)</td>
<td>211.0 (118.6)</td>
<td>9.4 (4.2)</td>
</tr>
<tr>
<td>*17/*3</td>
<td>1</td>
<td>0.25</td>
<td>0.12</td>
<td>13.7</td>
<td>10.7</td>
<td>0.31</td>
<td>167</td>
<td>1164</td>
<td>0.049</td>
<td>196.9</td>
<td>9.7</td>
</tr>
<tr>
<td>*17/*3</td>
<td>1</td>
<td>0.17</td>
<td>0.07</td>
<td>13.6</td>
<td>7.9</td>
<td>0.42</td>
<td>158</td>
<td>928</td>
<td>0.065</td>
<td>340.4</td>
<td>22.1</td>
</tr>
<tr>
<td>*1/*25</td>
<td>1</td>
<td>0.13</td>
<td>0.07</td>
<td>11.0</td>
<td>6.8</td>
<td>0.34</td>
<td>152</td>
<td>1216</td>
<td>0.059</td>
<td>240.4</td>
<td>14.2</td>
</tr>
<tr>
<td>*9/*26</td>
<td>1</td>
<td>0.03</td>
<td>0.18</td>
<td>6.6</td>
<td>2.3</td>
<td>0.29</td>
<td>210</td>
<td>1612</td>
<td>0.122</td>
<td>380.4</td>
<td>46.5</td>
</tr>
<tr>
<td>*4/*17</td>
<td>1</td>
<td>0.02</td>
<td>n/a</td>
<td>12.9</td>
<td>3.8</td>
<td>0.54</td>
<td>249</td>
<td>1644</td>
<td>0.210</td>
<td>154.5</td>
<td>32.4</td>
</tr>
<tr>
<td>All subjects</td>
<td>60</td>
<td>0.38 (0.25)</td>
<td>0.17 (0.11)</td>
<td>16.6 (4.6)</td>
<td>11.4 (4.2)</td>
<td>0.51 (0.21)</td>
<td>134 (37)</td>
<td>1052 (249)</td>
<td>0.086 (0.035)</td>
<td>221 (114)</td>
<td>18.8 (11.9)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Nicotine-d2 metabolite ratio (NMR-d2) quartile</th>
<th>Overall P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All subjects</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; Quartile</td>
</tr>
<tr>
<td>n</td>
<td>60</td>
<td>17</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>80.0 (15.2)</td>
<td>78.6 (14.9)</td>
</tr>
<tr>
<td><strong>A. Nicotine kinetics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CL&lt;sub&gt;NIC&lt;/sub&gt; (mL · min&lt;sup&gt;-1&lt;/sup&gt; · kg&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>16.6 (4.6)</td>
<td>14.2 (3.6)</td>
</tr>
<tr>
<td>Renal CL&lt;sub&gt;NIC&lt;/sub&gt; (mL · min&lt;sup&gt;-1&lt;/sup&gt; · kg&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.69 (0.59)</td>
<td>0.68 (0.45)</td>
</tr>
<tr>
<td>Nonrenal CL&lt;sub&gt;NIC&lt;/sub&gt; (mL · min&lt;sup&gt;-1&lt;/sup&gt; · kg&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>15.9 (4.5)</td>
<td>13.5 (3.7)</td>
</tr>
<tr>
<td>CL&lt;sub&gt;NIC → COT&lt;/sub&gt; (mL · min&lt;sup&gt;-1&lt;/sup&gt; · kg&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>11.4 (4.2)</td>
<td>8.9 (2.9)</td>
</tr>
<tr>
<td>Fractional conversion, f</td>
<td>0.68 (0.11)</td>
<td>0.62 (0.10)</td>
</tr>
<tr>
<td>Nicotine V&lt;sub&gt;ss&lt;/sub&gt; (L/kg)</td>
<td>2.6 (0.7)</td>
<td>3.0 (0.6)</td>
</tr>
<tr>
<td><strong>B. Cotinine kinetics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CL&lt;sub&gt;COT&lt;/sub&gt; (mL · min&lt;sup&gt;-1&lt;/sup&gt; · kg&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.51 (0.21)</td>
<td>0.39 (0.11)</td>
</tr>
<tr>
<td>Renal CL&lt;sub&gt;COT&lt;/sub&gt; (mL · min&lt;sup&gt;-1&lt;/sup&gt; · kg&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.10 (0.05)</td>
<td>0.10 (0.04)</td>
</tr>
<tr>
<td>Nonrenal CL&lt;sub&gt;COT&lt;/sub&gt; (mL · min&lt;sup&gt;-1&lt;/sup&gt; · kg&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.41 (0.19)</td>
<td>0.29 (0.12)</td>
</tr>
<tr>
<td>Cotinine t&lt;sub&gt;1/2&lt;/sub&gt; (min)</td>
<td>1053 (249)</td>
<td>1219 (256)</td>
</tr>
<tr>
<td>Cotinine V&lt;sub&gt;ss&lt;/sub&gt; (L/kg)</td>
<td>0.68 (0.16)</td>
<td>0.64 (0.18)</td>
</tr>
<tr>
<td>Plasma 3HC/COT&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.17 (0.11)</td>
<td>0.08 (0.02)</td>
</tr>
</tbody>
</table>

Notes: NMR-d2: 1<sup>st</sup> quartile, 0.11; median, 0.14; 3<sup>rd</sup> quartile, 0.19

<sup>a</sup>p<0.05, 3<sup>rd</sup> quartile significantly different from 1<sup>st</sup> quartile

<sup>b</sup>p<0.05, 4<sup>th</sup> quartile significantly different from 1<sup>st</sup> quartile

<sup>c</sup>p<0.05, 4<sup>th</sup> quartile significantly different from 2<sup>nd</sup> quartile

<sup>d</sup>p<0.05, 4<sup>th</sup> quartile significantly different from 3<sup>rd</sup> quartile

<sup>‡</sup>Plasma 3HC/COT is based on d2 analytes.
TABLE 3

Correlation between NMR and pharmacokinetic parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pearson Correlation with Fisher's z transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NMR(d₀)</td>
</tr>
<tr>
<td>Total CL₉₀C (mL · min⁻¹ · kg⁻¹)</td>
<td>0.52 *</td>
</tr>
<tr>
<td>Nonrenal CL₉₀C (mL · min⁻¹ · kg⁻¹)</td>
<td>0.51 *</td>
</tr>
<tr>
<td>Renal CL₉₀C (mL · min⁻¹ · kg⁻¹)</td>
<td>0.14</td>
</tr>
<tr>
<td>CL₉₀C → COT (mL · min⁻¹ · kg⁻¹)</td>
<td>0.59 *</td>
</tr>
<tr>
<td>Total CL₉₀F (mL · min⁻¹ · kg⁻¹)</td>
<td>0.75 *</td>
</tr>
<tr>
<td>Nonrenal CL₉₀F (mL · min⁻¹ · kg⁻¹)</td>
<td>0.79 *</td>
</tr>
<tr>
<td>Renal CL₉₀F (mL · min⁻¹ · kg⁻¹)</td>
<td>0.12</td>
</tr>
<tr>
<td>Nicotine T₁/₂ (min)</td>
<td>-0.65 *</td>
</tr>
<tr>
<td>Cotinine T₁/₂ (min)</td>
<td>-0.57 *</td>
</tr>
</tbody>
</table>

* P < 0.001
### TABLE 4

Nicotine and cotinine urine metabolites and metabolite ratios by CYP2A6 genotype activity

<table>
<thead>
<tr>
<th>CYP2A6 Genotype activity</th>
<th>n</th>
<th>Nicotine (NIC)</th>
<th>NIC-gluc</th>
<th>Cotinine (COT)</th>
<th>COT-gluc</th>
<th>3HC</th>
<th>3HC-gluc</th>
<th>Nicotine N-oxide (NNO)</th>
<th>Cotinine N-oxide (CNO)</th>
<th>Normal nicotine (NNIC)</th>
<th>Normal cotinine (NCOT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1/*1</td>
<td>37</td>
<td>26.0 (18.4)</td>
<td>4.6 (2.9)</td>
<td>20.0 (7.1)</td>
<td>3.8 (2.6)</td>
<td>152 (10.0)</td>
<td>27.2 (2.2)</td>
<td>248 (10.0)</td>
<td>1.6 (0.7)</td>
<td>0.7 (0.3)</td>
<td>0.7 (0.3)</td>
</tr>
<tr>
<td>*1/*17</td>
<td>8</td>
<td>17.2 (6.7)</td>
<td>4.1 (3.1)</td>
<td>24.2 (3.4)</td>
<td>2.6 (2.6)</td>
<td>134 (5.9)</td>
<td>45.4 (4.0)</td>
<td>304 (8.1)</td>
<td>1.8 (0.7)</td>
<td>0.8 (0.2)</td>
<td>1.0 (0.3)</td>
</tr>
<tr>
<td>*1/*9</td>
<td>2</td>
<td>10.4 (1.0)</td>
<td>5.9 (4.0)</td>
<td>23.0 (5.5)</td>
<td>4.8 (1.0)</td>
<td>163 (3.3)</td>
<td>33.3 (1.9)</td>
<td>328 (4.7)</td>
<td>1.8 (0.4)</td>
<td>0.9 (0.5)</td>
<td>0.8 (0.1)</td>
</tr>
<tr>
<td>*1/*24</td>
<td>1</td>
<td>11.6</td>
<td>1.9</td>
<td>20.5</td>
<td>0.8</td>
<td>250</td>
<td>62</td>
<td>287</td>
<td>3.0</td>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>*1/*4H</td>
<td>2</td>
<td>28.0 (21.8)</td>
<td>10.3 (2.5)</td>
<td>15.8 (3.3)</td>
<td>5.2 (1.8)</td>
<td>106 (7.8)</td>
<td>1.4 (1.5)</td>
<td>249 (7.7)</td>
<td>1.7 (0.8)</td>
<td>1.3 (0.8)</td>
<td>0.7 (0.6)</td>
</tr>
<tr>
<td>*1/*27</td>
<td>1</td>
<td>39.3</td>
<td>3.2</td>
<td>13.1</td>
<td>2.6</td>
<td>116</td>
<td>28</td>
<td>243</td>
<td>1.7</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>*1/*2</td>
<td>1</td>
<td>70</td>
<td>6.3</td>
<td>24.9</td>
<td>42</td>
<td>197</td>
<td>44</td>
<td>260</td>
<td>5.3</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>*1/*35</td>
<td>3</td>
<td>35.8 (4.8)</td>
<td>1.7 (0.2)</td>
<td>11.9 (3.6)</td>
<td>0.4 (0.6)</td>
<td>103 (6.7)</td>
<td>2.5 (2.6)</td>
<td>346 (10.2)</td>
<td>1.4 (0.4)</td>
<td>0.8 (0.2)</td>
<td>0.6 (0.3)</td>
</tr>
<tr>
<td>*17/*35</td>
<td>1</td>
<td>40</td>
<td>9.0</td>
<td>11.2</td>
<td>4.7</td>
<td>195</td>
<td>5.4</td>
<td>406</td>
<td>3.2</td>
<td>0.9</td>
<td>1.5</td>
</tr>
<tr>
<td>*17/*31</td>
<td>1</td>
<td>47.6</td>
<td>1.6</td>
<td>15.1</td>
<td>1.0</td>
<td>41</td>
<td>0.5</td>
<td>273</td>
<td>1.6</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>*1/*26</td>
<td>1</td>
<td>36.2</td>
<td>0.9</td>
<td>22.8</td>
<td>0.1</td>
<td>40</td>
<td>0.8</td>
<td>330</td>
<td>0.6</td>
<td>1.1</td>
<td>0.6</td>
</tr>
<tr>
<td>*9/*26</td>
<td>1</td>
<td>15.5</td>
<td>15.7</td>
<td>7.8</td>
<td>1.6</td>
<td>0.8</td>
<td>0.0</td>
<td>579</td>
<td>0.1</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>*4/*17</td>
<td>1</td>
<td>45.0</td>
<td>19.1</td>
<td>6.8</td>
<td>2.5</td>
<td>0.0</td>
<td>0.0</td>
<td>230</td>
<td>0.0</td>
<td>3.0</td>
<td>0.4</td>
</tr>
<tr>
<td>All subjects</td>
<td>60</td>
<td>24.9 (16.8)</td>
<td>5.0 (3.9)</td>
<td>19.5 (6.9)</td>
<td>3.3 (2.5)</td>
<td>140 (9.1)</td>
<td>2.9 (2.5)</td>
<td>27.4 (10.1)</td>
<td>1.6 (0.9)</td>
<td>0.8 (0.4)</td>
<td>0.7 (0.3)</td>
</tr>
</tbody>
</table>

**CYP2A6 Genotype activity**

**n** = number of subjects. Nicotine (NIC) and cotinine (COT) are expressed as μg/L. Nicotine N-oxide (NNO) and cotinine N-oxide (CNO) are expressed as μg/L. Normal nicotine (NNIC) and normal cotinine (NCOT) are expressed as μg/L.

**Pharmacogenet Genomics. Author manuscript; available in PMC 2017 July 01.**
<table>
<thead>
<tr>
<th>CYP2A6 Genotype activity</th>
<th>n</th>
<th>NIC-gluc/total NIC</th>
<th>COT-gluc/total COT</th>
<th>3HC-gluc/total 3HC</th>
<th>NIC-gluc/NIC</th>
<th>COT-gluc/COT</th>
<th>3HC-gluc/3HC</th>
<th>3HC/COT</th>
<th>[3HC + 3HC-gluc]/COT</th>
<th>Non C-oxidation</th>
<th>C-oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>*4/*17</td>
<td>1</td>
<td>29.8</td>
<td>26.9</td>
<td>0.0</td>
<td>42.5</td>
<td>36.8</td>
<td>0.00</td>
<td>0.00</td>
<td>87.2</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td>All subjects</td>
<td>60</td>
<td>21.1 (16.8)</td>
<td>14.8 (11.3)</td>
<td>15.5 (8.3)</td>
<td>35.0 (42.0)</td>
<td>19.9 (20.6)</td>
<td>19.5 (12.1)</td>
<td>0.76 (0.60)</td>
<td>0.90 (0.69)</td>
<td>57.2 (36.2)</td>
<td>42.8 (16.2)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Urine metabolite or ratio</th>
<th>Nicotine-d2 metabolite ratio (NMR-d2) quartile</th>
<th>Overall P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n 60 17 14 15 14</td>
<td></td>
</tr>
</tbody>
</table>

**A. Percent (%) of nicotine and metabolites recovered**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>All subjects</th>
<th>1st Quartile</th>
<th>2nd Quartile</th>
<th>3rd Quartile</th>
<th>4th Quartile</th>
<th>Overall P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine (NIC)</td>
<td>24.9 (16.8)</td>
<td>27.1 (15.0)</td>
<td>28.4 (19.5)</td>
<td>14.8 (8.1)</td>
<td>29.3 (19.8)</td>
<td>0.06</td>
</tr>
<tr>
<td>NIC glucuronide</td>
<td>5.0 (3.9)</td>
<td>5.7 (5.1)</td>
<td>5.4 (3.5)</td>
<td>4.6 (3.7)</td>
<td>4.1 (2.7)</td>
<td>0.67</td>
</tr>
<tr>
<td>Cotinine (COT)</td>
<td>19.5 (6.9)</td>
<td>18.0 (5.6)</td>
<td>18.6 (7.9)</td>
<td>22.2 (6.7)</td>
<td>19.1 (7.5)</td>
<td>0.35</td>
</tr>
<tr>
<td>COT glucuronide</td>
<td>3.3 (2.5)</td>
<td>2.3 (2.2)</td>
<td>3.6 (2.5)</td>
<td>3.1 (2.0)</td>
<td>4.5 (3.1)</td>
<td>0.11</td>
</tr>
<tr>
<td>3-Hydroxycotinine (3HC)</td>
<td>14.0 (9.1)</td>
<td>8.5 (5.2)</td>
<td>11.5 (6.7)</td>
<td>16.7 (6.6)</td>
<td>20.1 (12.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3HC glucuronide</td>
<td>2.9 (2.5)</td>
<td>1.8 (1.7)</td>
<td>2.2 (1.7)</td>
<td>4.3 (3.3)</td>
<td>3.4 (2.4)</td>
<td>0.02</td>
</tr>
<tr>
<td>Nicotine N-oxide (NNO)</td>
<td>27.4 (10.1)</td>
<td>33.5 (7.9)</td>
<td>27.1 (7.6)</td>
<td>30.6 (11.1)</td>
<td>16.8 (4.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cotinine N-oxide (CNO)</td>
<td>1.6 (0.9)</td>
<td>1.5 (0.8)</td>
<td>1.7 (1.3)</td>
<td>1.9 (0.7)</td>
<td>1.4 (0.7)</td>
<td>0.45</td>
</tr>
<tr>
<td>Nornicotine (NNIC)</td>
<td>0.8 (0.4)</td>
<td>1.0 (0.6)</td>
<td>0.8 (0.3)</td>
<td>0.8 (0.4)</td>
<td>0.5 (0.2)</td>
<td>0.07</td>
</tr>
<tr>
<td>Norcotinine (NCOT)</td>
<td>0.7 (0.3)</td>
<td>0.7 (0.3)</td>
<td>0.7 (0.4)</td>
<td>0.9 (0.3)</td>
<td>0.7 (0.3)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

**B. Nicotine and cotinine urine metabolite ratios**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>NIC-gluc/total NIC × 100 (%)</th>
<th>COT-gluc/total COT × 100 (%)</th>
<th>3HC-gluc/total 3HC × 100 (%)</th>
<th>NIC-gluc/NIC × 100 (%)</th>
<th>COT-gluc/COT × 100 (%)</th>
<th>3HC-gluc/3HC × 100 (%)</th>
<th>3HC/COT</th>
<th>[3HC + 3HC-gluc]/COT</th>
<th>Percent non C-oxidation (%)</th>
<th>Percent C-oxidation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21.1 (16.8)</td>
<td>14.8 (11.3)</td>
<td>15.5 (8.3)</td>
<td>35.0 (42.0)</td>
<td>19.9 (20.6)</td>
<td>19.5 (12.1)</td>
<td>0.76 (0.60)</td>
<td>0.90 (0.69)</td>
<td>57.2 (16.2)</td>
<td>42.8 (16.2)</td>
</tr>
</tbody>
</table>

**Notes:** NMR-d2: 1st quartile, 0.11; median, 0.14; 3rd quartile, 0.19

\(^a_p<0.05, 3^{rd}\) quartile significantly different from 1st quartile
\[ b \quad p<0.05, \text{4th quartile significantly different from 1st quartile} \]

\[ c \quad p<0.05, \text{4th quartile significantly different from 2nd quartile} \]

\[ d \quad p<0.05, \text{4th quartile significantly different from 3rd quartile} \]

Percent non C-oxidation is \( \frac{\text{NIC} + \text{NIC-gluc} + \text{NNO}}{\text{TNE}} \times 100 \)

Percent C-oxidation is \( \frac{\text{COT} + \text{COT-gluc} + \text{3HC} + \text{3HC-gluc} + \text{CNO} + \text{NNIC} + \text{NCOT}}{\text{TNE}} \times 100 \)
### TABLE 6

Correlation between NMR and urine metabolites and metabolite ratios

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pearson Correlation with Fisher’s <em>z</em> transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NMR(d₀)</td>
</tr>
<tr>
<td>Nicotine (NIC)</td>
<td>−0.01</td>
</tr>
<tr>
<td>NIC glucuronide</td>
<td>−0.24</td>
</tr>
<tr>
<td>Cotinine (COT)</td>
<td>0.001</td>
</tr>
<tr>
<td>COT glucuronide</td>
<td>0.24</td>
</tr>
<tr>
<td>3-Hydroxycotinine (3HC)</td>
<td>0.60     ***</td>
</tr>
<tr>
<td>3HC glucuronide</td>
<td>0.38     **</td>
</tr>
<tr>
<td>Nicotine N-oxide (NNO)</td>
<td>−0.60     ***</td>
</tr>
<tr>
<td>Cotinine N-oxide (CNO)</td>
<td>0.08</td>
</tr>
<tr>
<td>Nornicotine (NNIC)</td>
<td>−0.33     **</td>
</tr>
<tr>
<td>Norcotinine (NCOT)</td>
<td>0.11</td>
</tr>
<tr>
<td>NIC-gluc/total NIC</td>
<td>−0.11</td>
</tr>
<tr>
<td>COT-gluc/total COT</td>
<td>0.21</td>
</tr>
<tr>
<td>3HC-gluc/total 3HC</td>
<td>0.01</td>
</tr>
<tr>
<td>NIC-gluc/NIC</td>
<td>−0.11</td>
</tr>
<tr>
<td>COT-gluc/COT</td>
<td>0.21</td>
</tr>
<tr>
<td>3HC-gluc/6HC</td>
<td>0.01</td>
</tr>
<tr>
<td>3HC/COT</td>
<td>0.56     ***</td>
</tr>
<tr>
<td>[3HC + 3HC-gluc]/COT</td>
<td>0.57     ***</td>
</tr>
<tr>
<td>Non C-oxidation</td>
<td>−0.44     ***</td>
</tr>
<tr>
<td>C-oxidation</td>
<td>0.43     ***</td>
</tr>
</tbody>
</table>

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
# Statistical Report - Tobacco

## Reporting Period: December 2017

<table>
<thead>
<tr>
<th>Manufactured Domestically or Received from Puerto Rico</th>
<th>Current Month</th>
<th>Prior Month</th>
<th>Prior Year Current Month</th>
<th>Current Year Cumulative Year to Date</th>
<th>Prior Year Cumulative Year to Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarettes - Small</td>
<td>12,419,505,974</td>
<td>19,682,947,663</td>
<td>15,020,011,207</td>
<td>245,630,349,001</td>
<td>270,260,528,537</td>
</tr>
<tr>
<td>Cigarettes - Large</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cigars - Small</td>
<td>44,988,742</td>
<td>79,683,972</td>
<td>105,970,119</td>
<td>955,815,513</td>
<td>1,032,751,246</td>
</tr>
<tr>
<td>Cigars - Large</td>
<td>368,792,271</td>
<td>470,809,608</td>
<td>412,211,765</td>
<td>5,529,470,890</td>
<td>5,441,458,721</td>
</tr>
<tr>
<td>Snuff</td>
<td>8,688,556</td>
<td>9,974,421</td>
<td>9,056,463</td>
<td>120,223,566</td>
<td>120,498,770</td>
</tr>
<tr>
<td>Chewning Tobacco</td>
<td>1,156,882</td>
<td>7,296,237</td>
<td>1,207,570</td>
<td>23,499,403</td>
<td>18,954,435</td>
</tr>
<tr>
<td>Pipe Tobacco</td>
<td>2,517,562</td>
<td>2,944,372</td>
<td>2,622,951</td>
<td>36,502,414</td>
<td>36,824,320</td>
</tr>
<tr>
<td>Roll-Your-Own Tobacco</td>
<td>141,670</td>
<td>187,996</td>
<td>218,821</td>
<td>2,493,070</td>
<td>2,974,964</td>
</tr>
</tbody>
</table>

## 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                        |
| Chewning 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.                                         | 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.                                         | 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.                                         | 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.

http://www.ttb.gov
### Removed Tax Exempt - Smokeless Tobacco

<table>
<thead>
<tr>
<th>Current Month</th>
<th>Prior Month</th>
<th>Prior Year Current Month</th>
<th>Current Year Cumulative Year to Date</th>
<th>Prior Year Cumulative Year to Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snuff - Export &amp; To Export Warehouses</td>
<td>80,016</td>
<td>82,695</td>
<td>60,509</td>
<td>1,157,265</td>
</tr>
<tr>
<td>Other</td>
<td>686</td>
<td>791</td>
<td>812</td>
<td>14,560</td>
</tr>
<tr>
<td>Chewing Tobacco - Exp. &amp; To Exp. Whs.</td>
<td>0</td>
<td>3,086</td>
<td>1,620</td>
<td>29,127</td>
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<tr>
<td>Other</td>
<td>99</td>
<td>402</td>
<td>169</td>
<td>3,568</td>
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</table>

### Removed Tax Exempt - Pipe Tobacco

<table>
<thead>
<tr>
<th>Current Month</th>
<th>Prior Month</th>
<th>Prior Year Current Month</th>
<th>Current Year Cumulative Year to Date</th>
<th>Prior Year Cumulative Year to Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Export and To Export Warehouses</td>
<td>173,730</td>
<td>126,162</td>
<td>154,963</td>
<td>1,449,991</td>
</tr>
<tr>
<td>Other</td>
<td>216</td>
<td>354</td>
<td>235</td>
<td>2,200</td>
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### Removed Tax Exempt - Roll-Your-Own Tobacco

<table>
<thead>
<tr>
<th>Current Month</th>
<th>Prior Month</th>
<th>Prior Year Current Month</th>
<th>Current Year Cumulative Year to Date</th>
<th>Prior Year Cumulative Year to Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Export and To Export Warehouses</td>
<td>9,661</td>
<td>17,080</td>
<td>881</td>
<td>39,981</td>
</tr>
<tr>
<td>Other</td>
<td>7</td>
<td>4</td>
<td>5</td>
<td>191</td>
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</table>

### IMPORTED FROM FOREIGN COUNTRIES

Entered/Withdrawn for Consumption (As reported by the Bureau of the Census in Publication IM 146)

<table>
<thead>
<tr>
<th>Current Month</th>
<th>Prior Month</th>
<th>Prior Year Current Month</th>
<th>Current Year Cumulative Year to Date</th>
<th>Prior Year Cumulative Year to Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarettes, Total (USTSA 2402.20.1000, 2402.20.8000, 2402.20.9000)</td>
<td>633,143,000</td>
<td>557,526,000</td>
<td>758,366,000</td>
<td>7,866,487,000</td>
</tr>
<tr>
<td>Cigars - Small (USTSA 2402.10.3030, 2402.10.8030)</td>
<td>3,076,000</td>
<td>2,763,000</td>
<td>1,305,000</td>
<td>25,198,000</td>
</tr>
<tr>
<td>Cigars - Large: (USTSA 2402.10.3070, 2402.10.6000)</td>
<td>497,738,000</td>
<td>826,262,000</td>
<td>453,963,000</td>
<td>7,434,389,000</td>
</tr>
<tr>
<td>(USTSA 2402.10.8050, 2402.10.8080)</td>
<td>29,246,000</td>
<td>34,357,000</td>
<td>28,967,000</td>
<td>351,011,000</td>
</tr>
<tr>
<td>Total Large</td>
<td>526,984,000</td>
<td>860,619,000</td>
<td>482,930,000</td>
<td>7,785,400,000</td>
</tr>
<tr>
<td>Snuff (USTSA 2403.99.2040)</td>
<td>70,030</td>
<td>43,563</td>
<td>73,848</td>
<td>549,547</td>
</tr>
<tr>
<td>Chewing Tobacco (USTSA 2403.99.2030)</td>
<td>92,102</td>
<td>135,560</td>
<td>95,218</td>
<td>1,003,775</td>
</tr>
<tr>
<td>Pipe Tobacco (USTSA 2403.10.2020, 2403.10.2080)</td>
<td>507,246</td>
<td>48,019</td>
<td>432,770</td>
<td>3,665,466</td>
</tr>
<tr>
<td>Roll-Your-Own Tobacco (USTSA 2403.10.2050)</td>
<td>20,981</td>
<td>9,270</td>
<td>4,063</td>
<td>202,108</td>
</tr>
</tbody>
</table>

### Release to Domestic Factories Without Payment of Tax (Included also in above "Entered/Withdrawn for Consumption" Category)

<table>
<thead>
<tr>
<th>Current Month</th>
<th>Prior Month</th>
<th>Prior Year Current Month</th>
<th>Current Year Cumulative Year to Date</th>
<th>Prior Year Cumulative Year to Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarettes - Small</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>28,000</td>
</tr>
<tr>
<td>Cigarettes - Large</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cigars - Small</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cigars - Large</td>
<td>0</td>
<td>0</td>
<td>1,500</td>
<td>1,116</td>
</tr>
<tr>
<td>Snuff</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chewing Tobacco</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pipe Tobacco</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Roll-Your-Own Tobacco</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Onhand / Close of Business

<table>
<thead>
<tr>
<th>Current Month</th>
<th>Prior Month</th>
<th>Prior Year Current Month</th>
<th>Current Year Cumulative Year to Date</th>
<th>Prior Year Cumulative Year to Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarettes - Small</td>
<td>21,756,253,364</td>
<td>27,442,937,883</td>
<td>21,879,112,701</td>
<td>21,756,253,364</td>
</tr>
<tr>
<td>Cigarettes - Large</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cigars - Small</td>
<td>97,552,289</td>
<td>134,772,133</td>
<td>94,824,523</td>
<td></td>
</tr>
<tr>
<td>Cigars - Large</td>
<td>504,824,639</td>
<td>550,291,121</td>
<td>483,754,324</td>
<td></td>
</tr>
<tr>
<td>Snuff</td>
<td>3,476,462</td>
<td>3,920,544</td>
<td>4,065,912</td>
<td></td>
</tr>
<tr>
<td>Chewing Tobacco</td>
<td>665,213</td>
<td>756,850</td>
<td>613,098</td>
<td></td>
</tr>
<tr>
<td>Pipe Tobacco</td>
<td>1,658,254</td>
<td>1,922,533</td>
<td>1,623,696</td>
<td></td>
</tr>
<tr>
<td>Roll-Your-Own Tobacco</td>
<td>167,436</td>
<td>178,443</td>
<td>190,994</td>
<td></td>
</tr>
</tbody>
</table>

**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.

http://www.ttb.gov
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

Use of Tobacco Tax Stamps to Prevent and Reduce Illicit Tobacco Trade — United States, 2014

Jamie Chriqui, PhD1; Hillary DeLong, JD2; Camille Gourdet, JD2; Frank Chaloupka, PhD3; Sarah Matthes Edwards, MSPH4; Xin Xu, PhD4; Gabbi Promoff, MA4 (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
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,

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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

<table>
<thead>
<tr>
<th>State (and District of Columbia)</th>
<th>Cigarettes</th>
<th>LC and RYOT</th>
<th>Tribal stamping</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stamp required</td>
<td>Encrypted tax stamp</td>
<td>LC and/or RYOT taxed as a cigarette*</td>
</tr>
<tr>
<td>Alabama</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alaska</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arizona</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arkansas</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>California</td>
<td>Yes</td>
<td>Yes</td>
<td>RYOT</td>
</tr>
<tr>
<td>Colorado</td>
<td>Yes</td>
<td></td>
<td>LC</td>
</tr>
<tr>
<td>Connecticut</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delaware</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>District of Columbia</td>
<td>Yes</td>
<td></td>
<td>LC</td>
</tr>
<tr>
<td>Florida</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Georgia</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hawaii</td>
<td>Yes</td>
<td></td>
<td>LC</td>
</tr>
<tr>
<td>Idaho</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Illinois</td>
<td>Yes</td>
<td></td>
<td>LC</td>
</tr>
<tr>
<td>Indiana</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iowa</td>
<td>Yes</td>
<td></td>
<td>LC</td>
</tr>
<tr>
<td>Kansas</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kentucky</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Louisiana</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maine</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maryland</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Massachusetts</td>
<td>Yes</td>
<td>Yes</td>
<td>LC</td>
</tr>
<tr>
<td>Michigan</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Minnesota</td>
<td>Yes</td>
<td></td>
<td>LC</td>
</tr>
<tr>
<td>Mississippi</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Missouri</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Montana</td>
<td>Yes</td>
<td></td>
<td>LC</td>
</tr>
<tr>
<td>Nebraska</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nevada</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Hampshire</td>
<td>Yes</td>
<td></td>
<td>LC, RYOT</td>
</tr>
<tr>
<td>New Jersey</td>
<td>Yes</td>
<td>Yes††</td>
<td></td>
</tr>
<tr>
<td>New Mexico</td>
<td>Yes</td>
<td></td>
<td>LC, RYOT</td>
</tr>
<tr>
<td>New York</td>
<td>Yes</td>
<td></td>
<td>LC</td>
</tr>
<tr>
<td>North Carolina</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>North Dakota</td>
<td>No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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
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). Agreeing to comply with laws or regulations enforced by states might alleviate concerns about tax losses from tribal sales, because it

### 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

<table>
<thead>
<tr>
<th>State (and District of Columbia)</th>
<th>Stamp required</th>
<th>Encrypted tax stamp</th>
<th>LC and/or RYOT taxed as a cigarette*</th>
<th>LC and/or RYOT explicitly stamped</th>
<th>On-reservation tobacco sales require stamps on some or all products</th>
<th>Type of stamp(s) required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ohio</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td>Yes (all§)</td>
<td>SE, GT, TA</td>
</tr>
<tr>
<td>Oklahoma</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td>Yes (some††¶¶)</td>
<td>SE</td>
</tr>
<tr>
<td>Oregon</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pennsylvania</td>
<td>Yes</td>
<td>LC</td>
<td></td>
<td>LC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhode Island</td>
<td>Yes</td>
<td>LC</td>
<td></td>
<td>LC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Carolina</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Dakota</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tennessee</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Texas</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Utah</td>
<td>Yes</td>
<td>LC</td>
<td></td>
<td></td>
<td>Yes (some*††¶¶)</td>
<td>SE</td>
</tr>
<tr>
<td>Vermont</td>
<td>Yes</td>
<td>LC, RYOT</td>
<td></td>
<td>LC, RYOT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virginia</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washington</td>
<td>Yes</td>
<td>LC, RYOT</td>
<td></td>
<td>RYOT</td>
<td>Yes (all§)</td>
<td>SE, ST, TA</td>
</tr>
<tr>
<td>West Virginia</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wisconsin</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wyoming</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td>Yes (all§)</td>
<td>SE, GT</td>
</tr>
<tr>
<td>Totals</td>
<td>48</td>
<td>4</td>
<td>18</td>
<td>6</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>


**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).
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.

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.

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

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References

Approaches for Controlling Illicit Tobacco Trade — Nine Countries and the European Union

Hana Ross, PhD1; Muhammad Jami Husain, PhD2; Deliana Kostova, PhD2; Xin Xu, PhD2; Sarah M. Edwards, MSPH2; Frank J. Chaloupka, PhD3; Indu B. Ahluwalia, PhD2 (Author affiliations at end of text)

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.
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.

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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.


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-serving 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
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

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**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†**

<table>
<thead>
<tr>
<th>Company/event type</th>
<th>No. (%)</th>
<th>Fatality rate§</th>
<th>% Rate change¶</th>
<th>IRR</th>
<th>(95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Fatalities</td>
<td>1,189</td>
<td>25.0</td>
<td>-36.3</td>
<td>0.956</td>
<td>(0.932–0.980)</td>
<td>0.000**</td>
</tr>
<tr>
<td>By company (NAICS code)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Operators (211)</td>
<td>196 (16.5)</td>
<td>11.6</td>
<td>-58.2</td>
<td>0.917</td>
<td>(0.869–0.967)</td>
<td>0.001**</td>
</tr>
<tr>
<td>Drilling contractors (213111)</td>
<td>378 (31.8)</td>
<td>44.6</td>
<td>-27.2</td>
<td>0.969</td>
<td>(0.931–1.008)</td>
<td>0.118</td>
</tr>
<tr>
<td>Well-servicing companies (213112)</td>
<td>615 (51.7)</td>
<td>27.9</td>
<td>-33.4</td>
<td>0.960</td>
<td>(0.962–0.996)</td>
<td>0.028**</td>
</tr>
<tr>
<td>By event††</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transportation</td>
<td>479 (40.3)</td>
<td>10.1</td>
<td>-28.1</td>
<td>0.968</td>
<td>(0.938–0.998)</td>
<td>0.040**</td>
</tr>
<tr>
<td>Contact with objects/equipment</td>
<td>308 (25.9)</td>
<td>6.5</td>
<td>-60.8</td>
<td>0.910</td>
<td>(0.879–0.944)</td>
<td>0.000**</td>
</tr>
<tr>
<td>Fires/explosions §§</td>
<td>170 (14.3)</td>
<td>3.6</td>
<td>-41.3</td>
<td>0.948</td>
<td>(0.884–1.017)</td>
<td>0.137</td>
</tr>
<tr>
<td>Exposure to harmful environments/substances §§</td>
<td>104 (8.7)</td>
<td>2.2</td>
<td>-42.6</td>
<td>0.946</td>
<td>(0.890–1.006)</td>
<td>0.076</td>
</tr>
<tr>
<td>Falls §§</td>
<td>97 (8.2)</td>
<td>2.0</td>
<td>+26.8</td>
<td>1.024</td>
<td>(0.960–1.093)</td>
<td>0.469</td>
</tr>
</tbody>
</table>


**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.

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**TABLE 2. Annual fatality rates among workers in the oil and gas extraction industry, by company type and event type — United States, 2003–2013*†**

<table>
<thead>
<tr>
<th>Company/event type</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
<th>% Rate change§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>29.0</td>
<td>31.9</td>
<td>29.0</td>
<td>32.4</td>
<td>28.5</td>
<td>25.2</td>
<td>16.1</td>
<td>24.6</td>
<td>22.4</td>
<td>24.5</td>
<td>19.1</td>
<td>-36.3</td>
</tr>
<tr>
<td>By company</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Operators</td>
<td>14.1</td>
<td>23.9</td>
<td>13.5</td>
<td>16.3</td>
<td>10.3</td>
<td>13.1</td>
<td>7.5</td>
<td>7.6</td>
<td>7.6</td>
<td>13.8</td>
<td>6.1</td>
<td>-58.2</td>
</tr>
<tr>
<td>Drilling contractors</td>
<td>50.5</td>
<td>52.3</td>
<td>51.0</td>
<td>45.1</td>
<td>49.7</td>
<td>32.4</td>
<td>42.8</td>
<td>63.1</td>
<td>47.0</td>
<td>42.2</td>
<td>25.7</td>
<td>-27.2</td>
</tr>
<tr>
<td>Well-servicing companies</td>
<td>34.7</td>
<td>30.4</td>
<td>32.3</td>
<td>39.2</td>
<td>33.0</td>
<td>30.9</td>
<td>14.0</td>
<td>23.8</td>
<td>24.0</td>
<td>27.3</td>
<td>25.6</td>
<td>-33.4</td>
</tr>
<tr>
<td>By event</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transportation</td>
<td>10.6</td>
<td>14.7</td>
<td>10.1</td>
<td>11.2</td>
<td>11.7</td>
<td>10.3</td>
<td>6.4</td>
<td>9.4</td>
<td>10.2</td>
<td>11.0</td>
<td>7.5</td>
<td>-28.1</td>
</tr>
<tr>
<td>Contact with objects/equipment</td>
<td>8.9</td>
<td>9.5</td>
<td>8.0</td>
<td>10.1</td>
<td>9.4</td>
<td>6.3</td>
<td>5.0</td>
<td>4.8</td>
<td>5.2</td>
<td>4.3</td>
<td>4.3</td>
<td>-60.8</td>
</tr>
<tr>
<td>Fires/explosions §§</td>
<td>6.5</td>
<td>3.6</td>
<td>3.6</td>
<td>5.4</td>
<td>2.3</td>
<td>3.8</td>
<td>2.3</td>
<td>5.8</td>
<td>5.2</td>
<td>4.1</td>
<td>2.2</td>
<td>-41.3</td>
</tr>
<tr>
<td>Exposure to harmful environments/substances §§</td>
<td>1.7§</td>
<td>1.6§</td>
<td>3.3</td>
<td>3.1</td>
<td>3.5</td>
<td>2.5</td>
<td>2.1§</td>
<td>2.3</td>
<td>1.8§</td>
<td>1.4§</td>
<td>1.4§</td>
<td>-42.6</td>
</tr>
<tr>
<td>Falls §§</td>
<td>1.4§</td>
<td>2.6§</td>
<td>3.0</td>
<td>2.1§</td>
<td>1.4§</td>
<td>1.9§</td>
<td>0.7§</td>
<td>1.6§</td>
<td>2.0</td>
<td>3.2</td>
<td>2.4</td>
<td>+26.8</td>
</tr>
</tbody>
</table>


* 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.

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**


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**References**

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 (strept 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...
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.

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References

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 Staphylococcus 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, 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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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

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).

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* 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.