424 R&R and PHS-398 Specific
Table Of Contents

SF 424 R&R Face Page 2
Research & Related Other Project Information 3
Project Summary/Abstract 4
Project Narrative 5
Facilities And Other Resources 6
PHS 398 Cover Page Supplement 9
PHS 398 Research Plan 11
Specific Aims 12
Research Design and Methods 13
Protection Of Human Subjects 25
Inclusion Of Women And Minorites 28
Targeted/Planned Enrollment Table 29
Inclusion Of Children 30
Resource Sharing Plan(s) 31

PI: **Stepanov, Irina**	Title: Constituent yields and biomarkers of exposure for tobacco product regulation	
	FOA: PAR12-267	
	FOA Title: TOBACCO CONTROL REGULATORY RESEARCH - R01	
1 R01 CA179246-01		
	Organization: UNIVERSITY OF MINNESOTA	

RESEARCH & RELATED Other Project Information

1. * Are Human Subjects Involved? ☒ Yes ☐ No

    1.a If YES to Human Subjects

    Is the Project Exempt from Federal regulations? ☐ Yes ☒ No

    If no, is the IRB review Pending? ☒ Yes ☐ No

2. * Are Vertebrate Animals Used? ☐ Yes ☒ No

3. * Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4.a. * Does this project have an actual or potential impact on the environment? ☐ Yes ☒ No

5. * Is the research performance site designated, or eligible to be designated, as a historic place? ☐ Yes ☒ No

6. * Does this project involve activities outside of the United States or partnerships with international collaborators? ☐ Yes ☒ No

PROJECT SUMMARY

A mandated reduction in cigarette smoke of selected carcinogens and toxicants has been recommended by the World Health Organization Study Group on Tobacco Product Regulation (TobReg) and is now possible in the U.S. under the Family Smoking Prevention and Tobacco Control Act. Although a mandated reduction of individual toxicants and carcinogens may not necessarily lead to reduction in health risks, some potent carcinogens in cigarette smoke can be substantially reduced by modifying cigarette manufacturing approaches. Therefore, the overall goal would be to progressively reduce levels of these constituents in mainstream smoke as measured by standardized machine determined methods. However, the issue of how to test and regulate the contents of cigarette smoke represents a critical challenge. The currently used standard machine testing methods do not account for the complexities of smoker-cigarette interaction and are widely recognized to be inadequate for the prediction of human exposures. TobReg study group recommended that levels of toxicants be established per mg of nicotine. However, it is not known how the constituent per mg nicotine emissions in cigarette smoke are related to individual constituent exposures in smokers, and which factors may affect this relationship. Moreover, it is not clear which of the traditionally used standard smoking machine regimens may deliver constituent per mg nicotine levels in the U.S. cigarettes that are most closely related to the smokers' exposure. The goal of our proposal is to address these critical gaps. Building on our expertise in the analysis of tobacco products and biomarkers of exposure, we will focus on the carcinogenic tobacco-specific nitrosamines *N'*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to conduct research as described in the following specific aims: (1) To examine NNN and NNK per mg nicotine emissions in various U.S. cigarette brands under different smoking regimens. (2) To examine the extent to which changes in NNN and NNK per mg nicotine yields in smoke succeed in predicting changes in smokers' exposure to these carcinogens. (3) To determine which individual factors (for example, duration and intensity of smoking, nicotine metabolism, demographics) may affect the relationship between the machine-measured TSNA per mg nicotine and exposures in smokers. These factors may need to be considered when examining constituent/mg nicotine smoke yields for regulatory purposes. This research is critical for expanding the science base that informs the FDA as it develops, evaluates, and implements tobacco product regulations.

PROJECT NARRATIVE (Relevance to Public Health)

This proposal addresses several research priorities related to the regulatory authority of the Food and Drug Administration (FDA) Center for Tobacco Products as mandated by the Family Smoking Prevention and Tobacco Control Act. Scientific evidence supports the important role of tobacco and cigarette smoke carcinogens in the development of cancers associated with cigarette smoking. Regulation of the levels of harmful constituents in cigarette smoke is one of the tobacco control strategies that now can be employed by the FDA and may serve to reduce tobacco carcinogen exposures in those smokers who are unable or unwilling to quit smoking. Such regulation will require a valid and robust approach to the assessment of comparative toxicity and carcinogenicity among various cigarette brands. This proposal will help develop a testing approach that can produce meaningful predictions of changes in human exposure due to changes in constituent levels in cigarette smoke, and hence serve as a reliable measure for product regulation. Thus, the proposed research will generate findings and data that are directly relevant to inform the FDA's regulation of the manufacture, distribution, and marketing of tobacco products to protect public health.

FACILITIES AND OTHER RESOURCES

Laboratory:

All the laboratory analyses will be conducted at the Masonic Cancer Center, University of Minnesota. Currently, laboratory space available for the implementation of the proposed research is located at the Masonic Cancer Center Building (MCRB) and consists of 1) ~2500 sq ft of laboratory equipped with 62 linear ft of fume hood work area as well as standard lab benches and equipment space; 2) shared use with others on the floor of ~2000 sq ft of shared core space which includes two coldrooms, a darkroom, large shared equipment space, 500 sq ft for mass spectrometry and small 150 sq ft laboratories for specialized applications; 3) free access to each of the other three research floors and their equipment. This space will expand as we move to a new research building in June 2013. The building is part of a complex of research facilities that is being constructed on the University of Minnesota Twin Cities campus and will offer 700,000 square feet of research space. Principal investigators moving to the new building, including Dr. Stepanov, will be given large laboratory space in a building that is designed to foster interdisciplinary collaborations. In the new building, the mass spectrometry facility will occupy 1800 ft^2 of new, specially designed dedicated space.

Clinical:

The proposed study will be conducted at the University of Minnesota's Tobacco Research Programs housed at the Delaware Clinical Research Unit at 717 Delaware St. SE Minneapolis, MN. Dr. Dorothy Hatsukami serves as the Director for this Program. Currently, the University of Minnesota Tobacco Research Programs administers the 10 R01s, 1 R-23, 1 PPG project, 1 P50 project, an NCI contract and two cooperative agreements. We have a Research Projects Coordinator (Joni Jensen, MPH) who oversees all research and is responsible for logistics of implementing the protocols and standard operating procedures. She is also responsible for the quality control of the projects by ensuring that all studies follow ethical scientific standards and that procedures meet GCP standards, that all regulatory forms are completed including Investigational New Drug forms and Institutional Review Board applications, and that the DSMB process is in place. Ms. Jensen has been working in this capacity for over 20 years and is a Certified Clinical Research Coordinator. We also have an Administrator (Kathy Longley) who ensures the smooth operation of the daily activities of the Program. In addition, the Program has a registered nurse practitioner, 14 research project coordinators, and two undergraduate research assistants. The shared space at the Delaware Clinical Research Units includes a shared waiting room with a receptionist, 7 physical exam rooms (two dedicated to the Tobacco Research Programs), 1 phlebotomy room, 5 interview rooms, 2 day hospital rooms, an infusion room, 1 smoking laboratory with one way observation room, laboratory space for processing blood, urine processing laboratory, a locked medication supply room, locked protocol room for subject files, cubicles for data entry, management and analyses, locked supply storage and access to three conference rooms. Two restrooms are in the clinical space for urine collections. We have dedicated space for our biorepository with key card access containing ten -20 freezers. We also have access to all of the resources of the University of Minnesota for our use, as needed.

Animal:

n/a

Computer:

Both Macintosh and IBM-type personal computers are networked in the MCRB via a common server. Shared hardware includes network printers (HP LaserJet 5SiMX). Standard software supported includes Word, Excel, Powerpoint, and Access. The Tobacco Research Programs is also fully equipped with PC computers that are networked to a secure server and laser printers.

Office:

Dr. Stepanov's office with desk, files, shelves, etc. and telephone and computer networking capability, is located in the MCRB and has direct access to all laboratories and equipment. Office spaces for the Dorothy Hatsukami and Joni Jensen and other clinical staff are located in 717 Delaware St. SE. A full range of secretarial and office resources are also available to support the research including PC computers with laser printers, copy machine, fax machine, etc. Office space is also available to the students. All necessary word processing, email, statistical and graphics software is available.

Other:

Laboratory glassware cleaning and sterilization is provided by a staffed facility in the Masonic Cancer Center. The MCRB also includes a 150-seat fully equipped seminar room as well as access to six conference rooms, copy and fax machines. Clinical and office facilities have laser printers, a secure server, scanner, copying machines and FAX machines.

EQUIPMENT

Major equipment available for the laboratory analyses includes high performance liquid chromatography (HPLC) units with associated detectors, including radioflow, diode array, and fluorescence. Gas chromatographs (GC) with flame ionization and electron capture detectors as well as nitrosamine specific/nitrogen detector (Thermedics TEA). Mass spectrometry equipment includes a Finnigan MAT TSQ-7000 GC/LC tandem mass spectrometer, an Agilent 5973 GC-mass selective detector, several ThermoFinnigan TSQ-Quantum mass spectrometers, two TSQ-Vantage mass spectrometers, and an LTQ-Orbitrap mass spectrometer. Other equipment includes ultracentrifuge plus rotors, UV spectrophotometer, analytical balances, toploading balances, rotavaps, personal computers, vacuum pumps, integrators, freezers. NMR is available through the Department of Medicinal Chemistry and the University High Field NMR facility which is equipped with 300, 500, 600, and 800mHz spectrometers. Additional equipment is also available at the clinical site and includes: defibrillator, emergency medical cart, oxygen, spirometry machine, CO monitors, electronic weight scales, and Dinamap blood pressure monitors.

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

1. Project Director / Principal Investigator (PD/PI)

Prefix:
* First Name: Irina
Middle Name:
* Last Name: Stepanov
Suffix:

2. Human Subjects

Clinical Trial? ☒ No ☐ Yes

* Agency-Defined Phase III Clinical Trial? ☐ No ☐ Yes

3. Applicant Organization Contact

Person to be contacted on matters involving this application

Prefix:
* First Name: Derek
Middle Name:
* Last Name: Krogstad
Suffix:

* Phone Number: 612-624-5599
Fax Number: 612-624-4843
Email: awards@umn.edu

* Title: Grant and Contract Administrator

* Street1: 200 Oak St. SE, Suite 450
Street2:
* City: Minneapolis
County/Parish: Hennepin
* State: MN: Minnesota
Province:
* Country: USA: UNITED STATES
* Zip / Postal Code: 55455-2070

PHS 398 Cover Page Supplement

4. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? ☒ No ☐ Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/research/registry/. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Cell Line(s): ☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page. The response provided on that page, regarding the type of application being submitted, is repeated for your reference, as you attach the appropriate sections of the Research Plan.

*Type of Application:

☒ New ☐ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

1. SPECIFIC AIMS

A mandated reduction in cigarette smoke of selected carcinogens and toxicants has been recommended by the World Health Organization Study Group on Tobacco Product Regulation (TobReg)[1] and is now possible in the U.S. under the Family Smoking Prevention and Tobacco Control Act.[2] As an initial step, the Food and Drug Administration (FDA) has identified a list of harmful and potentially harmful constituents that have to be disclosed by tobacco manufacturers.[3] Over time, this information will assist the FDA in developing standards for these constituents in cigarette smoke. However, the issue of how to test and regulate the contents of cigarette smoke represents a critical challenge. The currently used standard machine testing methods do not account for the complexities of smoker-cigarette interaction and are widely recognized to be inadequate for the prediction of human exposures.[1] Attempts to develop a single smoking regimen that is representative of human smoking behavior have not been successful.[4] Therefore, there is an urgent need to develop a testing approach that can produce meaningful predictions of changes in human exposure due to changes in constituent levels in cigarette smoke, and hence serve as a reliable measure for product regulation.

The TobReg study group recommended that levels of constituents be established per milligram (mg) of nicotine.[1] This approach is a promising solution for regulatory purposes: being still based on machine testing and thus allowing for standardized comparisons among brands, it nonetheless shifts away from attempts to reproduce human smoking behavior and towards characterization of product toxicity. However, it is not known how the constituent per mg nicotine emissions in cigarette smoke are related to the constituent exposures in smokers, and which factors may affect this relationship. Moreover, it is not clear which of the traditionally used standard smoking machine regimens may deliver constituent per mg nicotine levels in the U.S. cigarettes that are most closely related to the smokers' exposure. The goal of our proposal is to address these critical gaps.

Building on our extensive expertise in the analysis of tobacco products and biomarkers of exposure, we will focus on *N'*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). These carcinogenic tobacco-specific nitrosamines are thought to be causative agents for the development of several tobacco-induced cancers,[5-7] and are among the constituents targeted by the FDA.[2,3] We recently demonstrated a substantial variation in NNN and NNK levels among regular "full flavor" U.S. cigarette brands that do not differ considerably in nicotine content.[8,9] An international comparison demonstrated that smokers' exposure to NNK varies significantly by countries in which cigarettes with differing levels of this carcinogen are smoked.[10] Moreover, our previous brand-switching study showed a reduction in urinary biomarkers of exposure to NNK when smokers temporarily switched to a cigarette brand with reduced TSNA, but similar nicotine, content compared to their usual brand.[11] Together, these studies suggest that differences in the machine-measured TSNA/mg nicotine content across U.S. cigarette brands may lead to differential exposure to NNN and NNK among U.S. smokers. Our preliminary data support this hypothesis.

Primary specific aims in the proposed study are:

1. <u>To examine NNN and NNK per mg nicotine emissions in the smoke of various U.S. cigarette brands under different smoking machine regimens.</u> In this aim, we will determine whether the existing standard smoking machine regimens produce different TSNA per mg nicotine yields in U.S. cigarettes.
2. <u>To examine the extent to which differences in smoke yields of NNN and NNK per mg nicotine are predictive of differences in smokers' exposure to these carcinogens.</u> In this aim, we will assess biomarkers of exposure in habitual smokers of cigarettes with differing TSNA per mg nicotine smoke yields, as established in Aim 1. We will also explore whether the TSNA per nicotine content in spent cigarette filters can serve as a robust and non-invasive proxy for smokers' TSNA per mg nicotine intake.

Secondary specific aim in the proposed study is:

3. <u>To determine which individual factors may affect the relationship between the machine-measured TSNA per mg nicotine and exposures in smokers.</u> Such factors as duration and intensity of smoking, nicotine metabolism, and demographics may need to be considered when examining constituent/mg nicotine smoke yields for regulatory purposes.

In summary, the proposed research will test the hypothesis that changes in TSNA per mg nicotine yields in cigarette smoke are better predictors of changes in smokers' exposure as compared to absolute (per cigarette) emissions. This type of research is critical for expanding the science base that informs the FDA as it develops, evaluates, and implements tobacco product regulation programs.

2. RESEARCH STRATEGY

A. SIGNIFICANCE

Importance of tobacco constituent regulation

Tobacco use is a particularly complex public health problem. It encompasses a wide range of issues related to addiction, social interactions, environmental influences, individual health risks, and population effects. Consequently, the FDA's tobacco product regulation strategy incorporates a variety of aspects related to tobacco product manufacture, distribution, and marketing. And due to their crucial role in tobacco addiction and toxicity, chemical constituents of tobacco and cigarette smoke are central to many of the issues related to tobacco product regulation. FDA established a list of 93 harmful and potentially harmful constituents in tobacco and cigarette smoke, based on the available evidence for their ability or potential to cause serious health problems including cancer, lung disease, and addiction to tobacco products.[12] According to the Family Smoking Prevention and Tobacco Control Act, the FDA is allowed to set standards for these constituents "as appropriate to protect public health".[2] The list of harmful and potentially constituents includes numerous chemical carcinogens that are believed to be major contributors to the risk of developing smoking-induced cancers.[12] At present, there is no evidence that a reduction of an individual cancer-causing constituent in cigarette smoke will lead to a reduction in cancer risk in smokers. However, the levels of some potent carcinogens in cigarette smoke can be substantially reduced by modifying cigarette manufacturing approaches.[13] Therefore, those smokers who are unable or unwilling to quit, are being unnecessary exposed to unjustifiably high levels of certain human carcinogens. Regulation of tobacco constituents will help to minimize these exposures.

Tobacco-specific *N*-nitrosamines

Among the carcinogenic tobacco constituents that can be reduced in tobacco products and are targeted by the FDA are the tobacco-specific nitrosamines (TSNA) *N′*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Figure 1).[3] These carcinogens are among the first constituents previously proposed for regulation and future reduction by the TobReg study group.[1,14] Based on the extensive scientific evidence, NNN and NNK are thought to be causative agents for the development of cancers of the lung, pancreas, oral cavity, and esophagus in smokers, and are classified by the International Agency for Research on Cancer as Group 1 carcinogens (carcinogenic to humans).[5-7] These nitrosamines are formed from tobacco alkaloids during tobacco processing, and the amounts that are formed depend on tobacco type, nitrate content, and tobacco processing techniques.[5] The amounts of TSNA formed during cigarette burning are not significant and the levels of TSNA in tobacco were shown to determine smoke yields.[6,15-17] Therefore, changes in cigarette manufacturing practices can substantially reduce, or nearly eliminate, TSNA levels in cigarette smoke. This is clearly demonstrated by the significant variation of TSNA levels across countries and the type of cigarettes,[10,16] and by the reported reductions in TSNA levels in Canadian cigarettes.[18] We recently demonstrated that the levels of TSNA also vary significantly among the U.S. cigarettes (Table 1).[8] The lowest levels of NNN and NNK were found in Marlboro Virginia Blend – a brand that is made with Virginia bright tobacco known to form low amounts of TSNA.[19] These low levels of TSNA are not usual for a U.S. cigarette brand. However, even if this particular brand is not taken into account, the levels of NNN and NNK in products listed in Table 1 vary about 3-fold. These observations further underline the need for the regulation of these potent carcinogens in U.S. cigarettes.

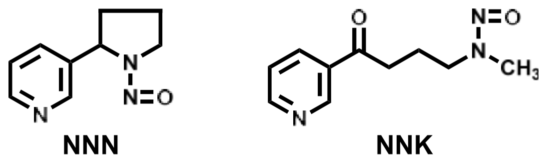

Figure 1. Structures of *N′*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

Table 1. NNN and NNK in smoke of U.S. cigarettes.[8]

Cigarette brand*	ng/cigarette	
	NNN	NNK
Marlboro Full Flavor	171.0	90.3
Marlboro Special Blend	141.6	77.3
Marlboro Blend # 27	145.2	91.2
Marlboro Blend # 54	232.1	133.7
Marlboro Smooth Menthol	164.2	86.4
Marlboro Virginia Blend	19.5	25.6
Basic Full Flavor	207.1	146.1
Newport Menthol	151.8	65.6
Camel Full Flavor	120.2	67.5
Camel # 9	102.8	44.4
Camel # 9 Menthol	75.2	43.4
Camel Silver	100.3	45.6
Camel Crush	96.9	48.1
Winston Full Flavor	172.8	78.5
Kool Filter Kings	135.8	63.0
Pall Mall Full Flavor	114.3	72.8
Doral Full Flavor	225.9	100.4

* All brands are king size, hard packs.

It should be noted that there is a common concern that setting limits for TSNA levels in cigarette smoke may increase smokers' exposure to another important group of carcinogens – polycyclic aromatic hydrocarbons (PAH). For example, international cigarette brands generally deliver increased amounts of PAH as TSNA levels decrease.[20] This inverse correlation was also observed upon comparison of the mainstream smoke from

individual types of tobaccos known to differ in TSNA content.[21] This relationship is due to the contrasting effect of nitrate content in tobacco on TSNA formation and PAH pyrosynthesis. Higher nitrate content leads to the formation of larger TSNA amounts during tobacco processing, but also generates higher amounts of nitrogen oxides during tobacco combustion; these oxides 'capture' and neutralize some radicals that otherwise would form PAH (45). However, brand-by-brand examination of the international sample of cigarettes for which overall negative correlation between TSNA and PAH was observed shows that many individual brands do not follow this pattern (43). This observation suggests that there are technologies available to reduce TSNA levels in cigarette smoke without increasing the amounts of pyrosynthesized PAH. In support of this notion, our preliminary analysis did not detect any significant relationship between the TSNA and PAH levels in a limited set of U.S. cigarettes. While this is not our primary goal in the proposed study, we will apply our recently developed robust procedure[22] to analyze a range of carcinogenic PAH in cigarette brands tested in Specific Aim 1. Understanding of the relationship between the TSNA and PAH levels in the smoke of various brands of U.S. cigarettes and the corresponding exposures in smokers can provide important information for the development by the FDA of standards setting maximum allowable levels for these carcinogens in cigarette smoke.

Smoking regimens and constituent yields

The measurements of cigarette smoke constituents for regulatory purposes have to rely on smoking machine-based methods. However, the smoker-cigarette interaction is much more complex than any single machine-based regimen.[23] It is primarily driven by a smoker's pursuit of nicotine – the main known addictive constituent in tobacco and cigarette smoke and the reason why people use tobacco products.[24,25] To control their nicotine intake, smokers adjust puff size, duration, frequency, and depth of inhalation, which ultimately affects their exposure to other constituents present in cigarette smoke. Smokers also regulate their nicotine intake by blocking filter ventilation holes, which reduces cigarette smoke dilution with air.[26] Therefore it is not surprising that the standardized machine-measured 'per cigarette' yields have been found to be unreliable predictors of the actual constituent intake by smokers.[23,27-30] Previous marketing of "light" cigarettes is an example of the poor predictive value of the machine-measured yields. The cigarettes – currently banned from being called "light" – are designed to include several elements that reduce the smoking machine-measured tar and nicotine yields. However, because smokers increase their smoking intensity in response to the reduced nicotine content in smoke, these cigarettes did not reduce smokers' exposure to tobacco carcinogens and did not lower the risk of smoking-induced diseases.[31-33] Since the commonly used standard smoking machine methods have been shown to underestimate human exposures, several alternative machine testing regimens have been tested in attempts to mimic smoking in humans, but none was shown to be representative of human smoking behavior.[4]

A promising strategy for overcoming the disadvantages of the machine-based measurements has been proposed by the TobReg Study Group, which has recommended to change the interpretation of these measurements by normalizing cigarette smoke constituent levels per milligram (mg) of nicotine.[1,14] The benefit of this strategy is that while it still relies on the smoking machine-generated constituent levels, it does not depend on either the dilution of the smoke or the volume of smoke per cigarette, and thus allows for adequate comparisons of smoke toxicity among various cigarette brands. Therefore, this approach could be potentially used for regulatory purposes by the FDA, instead of its current recommendation that the constituent amounts be measured 'per cigarette'.[3]

What is not clear, however, is which smoking machine regimen should be used to measure constituent per mg nicotine yields in cigarette smoke. Different smoking machine regimens produce different total volumes of smoke, and subsequently different constituent yields, per cigarette.[35] The commonly used machine-smoking regimen in the U.S. was the Federal Trade Commission (FTC) method – an adaptation of the International Organization for Standardization (ISO) which draws 35 mL puff volumes over 2 s, with 60 s intervals between puffs.[36] Other commonly used regimens are more intense: the one used in the Massachusetts Benchmark

Study draws 45 ml puff volumes and partially blocks filter ventilation holes, and the one developed by Health Canada (Canadian intense) draws 55 ml puff volumes and completely blocks filter vents, both regimens drawing puffs at higher frequency than the FTC/ISO method.[37-39] These changes lead to well-documented differences in 'per cigarette' constituent yields among the three smoking regimens, with the yields increasing in the order FTC < Massachusetts < Canadian intense.[6,34] For example, yields of nicotine, TSNA, and BaP in cigarette smoke can be 2–4 times higher under Canadian intense regimen than under FTC smoking parameters.[6] Based on these findings, the TobReg has suggested using Canadian intense method for measuring 'per nicotine' yields in cigarette smoke.[14] However, constituent 'per nicotine' yields may be affected by smoking conditions differently from the 'per cigarette' yields. For example, the NNK per mg nicotine yields of some Canadian cigarettes tested under the Canadian method ranged from 29% lower to 63% higher than under the FTC/ISO regimen, depending on cigarette brand.[40] Analysis of data reported by Counts et al. shows that NNK per mg nicotine yields change by as much as 240% across smoking regimens for some international brands.[34] The information on constituent per mg nicotine yields in individual U.S. cigarette brands smoked at different smoking regimens is limited. Analysis of the U.S. cigarette brands from the report by Counts et al.[34] shows that per nicotine emissions of NNN and NNK generally increase in the order Canadian intense < Massachusetts < FTC, which is opposite of the order in which 'per cigarette' emissions increase (Table 2). Currently, the FDA's Draft Guidance for Industry recommends that the constituent amounts in cigarette smoke be reported for two different smoking regimens: non-intense and intense.[3] Information on how smoking regimens of different intensity affect the constituent per mg nicotine emissions in various U.S. cigarette brands would provide the FDA with an important tool for comparisons of cigarette toxicity and developing standards for product performance.

Table 2. NNN and NNK yields per mg nicotine in some U.S. cigarette brands.[34]

Sample ID*	yield, ng/mg nicotine FTC	Mass.	Intense
NNN			
E5	153.9	145.3	151.3
E15	179.8	165.0	162.5
E27	225.0	194.4	175.8
E28	194.1	179.7	157.8
E30	174.7	157.7	146.0
V5	158.6	138.2	132.8
V9	292.3	208.8	185.8
NNK			
E5	105.7	104.3	111.1
E15	106.6	101.5	109.1
E27	117.2	108.1	99.7
E28	112.4	105.3	94.7
E30	118.7	110.3	91.0
V5	109.3	96.8	98.0
V9	147.7	87.8	86.3

* A mixed set of U.S. filtered cigarettes, includes regular, "light", and "ultralight" varieties

In addition to the comparative evaluation of cigarette toxicity among various cigarette brands, a critical question is how the constituent per mg nicotine emissions in cigarette smoke are related to exposures in smokers. In other words, are the constituent per mg nicotine yields better predictors of the smokers' exposures than the 'per cigarette' yields, which were shown not to represent actual constituent intakes in smokers? Addressing this research gap is fundamental to the understanding of whether the 'per nicotine' constituent yields are more relevant to human exposures than the 'per cigarette' emissions, and thus should be adopted by the FDA as an approach for establishing performance standards.

Relationship between constituent yields and biomarkers

Biomarkers of exposure to cigarette smoke constituents account for a variety of factors that affect constituent intake by smokers, including the features of the cigarette (for example filter ventilation) and smoking intensity, and can provide valuable information on the uptake of individual constituents by individual smokers. Therefore, tobacco constituent biomarkers can be used as a powerful tool in tobacco product regulation.[41]

Human exposure to NNN and NNK can be assessed via the measurement of urinary total NNN and total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), the sum of free and glucuronidated NNN and NNAL, respectively.[42] Urinary levels of total NNAL have been shown to increase with an increase in the number of cigarettes smoked per day (CPD),[43,44] and decrease with reduced smoking or smoking cessation.[45,46] Moreover, an international comparison revealed the relationship between urinary total NNAL and smoke NNK levels: urinary total NNAL was significantly lower among Canadian smokers compared to U.S. smokers, which is consistent with the significantly lower TSNA levels in Canadian cigarettes as compared to the most popular U.S. brands.[10] However, no studies have been conducted to evaluate the extent of variation in exposure to NNN and NNK among U.S. smokers due to customary smoking of cigarette brands that differ in TSNA content. Previous evidence of similar exposure to NNK between smokers of regular and "light" cigarettes does not answer this question, because "tar" values that were used to categorize cigarettes into regular and "light" may correlate poorly with certain smoke constituents, including TSNA.[23,47]

We conducted a preliminary analysis of the potential differences in biomarker levels between smokers of Marlboro and Camel cigarettes that differ in NNK per mg nicotine.[8,48] We merged demographics, smoking

history, and urinary total NNAL data from 8 studies of adult smokers that were carried out by our group, selecting smokers of regular full flavor Marlboro and Camel cigarettes. After adjustment for demographics and cigarettes per day, smokers of Camel cigarettes had significantly lower total NNAL levels, both when adjusted for creatinine (p=0.0482) and the nicotine biomarker cotinine (p=0.0441) levels, compared to smokers of Marlboro cigarettes. Even though the statistical significance is marginal in this limited preliminary analysis, these results support the hypothesis that the differences in constituent per mg nicotine yields among the U.S. cigarette brands may lead to the corresponding differences in the constituent uptake by smokers of these cigarettes. This type of information is of particular interest because of the observed significant dose-dependent association between prospectively measured urinary total NNAL and the risk of lung cancer in U.S. smokers.[49] This association was also found in prospective cohorts of smokers from Singapore and Shanghai, China.[50] In addition, our recent study demonstrated that urinary total NNN is a strong predictor of esophageal cancer in smokers.[51]

Our preliminary data also suggest that the measurement of the amounts of constituents that accompany nicotine intake by smokers might be a viable alternative, or a supplemental assessment, to the measurement of the overall exposure levels. More specifically, similar to the measurement of constituent per nicotine yields in cigarette smoke, the levels of biomarkers of exposure to these constituents can be expressed per biomarkers of nicotine intake. Total nicotine exposure can be measured by analyzing urinary total nicotine equivalents – the sum of biomarkers that account for up to 95% of the nicotine dose, including nicotine, nicotine-*N*-glucuronide, cotinine, cotinine-*N*-glucuronide, *trans*-3'-hydroxycotinine (3-HC), and 3-HC- glucuronides.[52] Since nicotine is the major "driver" of smoking intensity, the expression of the molar amounts of biomarkers per nicotine equivalents will provide a measure of the constituent intake that accompanies nicotine doses in smokers, independent of the differences in smoking intensities.

Analysis of spent cigarette filters as a proxy for constituent intake

While biomarkers of exposure are crucial for the measurements of tobacco constituent intake in smokers, biological sample collection may be invasive and analytical procedures for their measurements may be labor-intensive. These disadvantages are not as essential at the exploratory stages of research. However, routine measurements in situations when a rapid and robust assessment is desired, as it can be expected in regulatory applications, would greatly benefit from a more easily accessible and manageable proxy measure. For example, analysis of cigarette filters has been employed by some researchers as a proxy for smoking behavior and exposure to tobacco smoke constituents.[53] Analysis of nicotine in filters has been extensively used by various tobacco industry researchers for comparisons of consumer-smoked , or 'mouth-level' cigarette yields with tar and nicotine emissions in cigarette smoke, and generally produced a good agreement between the two types of yields.[53-55] The industry researchers also reported a strong correlation between nicotine levels in cigarette filter with salivary and urinary nicotine metabolites in smokers,[56,57] but no consistent relationship between these measures and ISO tar yields.[57] A study in Germany showed a significant correlation between filter nicotine and urinary biomarkers of exposure to several tobacco constituents, including TSNA.[58] Similarly, the study of TSNA exposure among smokers living in countries where cigarettes with differing levels of these carcinogens are produced showed that mouth-level exposure to TSNA, as measured by the amount of a tobacco-specific compound solanesol in cigarette filters, correlated with the levels of these carcinogens in cigarette smoke and with urinary biomarkers of exposure.[10] Together, these studies suggest that analysis of TSNA per mg nicotine content in spent cigarette filters might serve as an accurate and robust estimate of TSNA per mg nicotine intake in smokers of these cigarettes.

Individual differences

The extent of tobacco constituent exposure in smokers can be influenced by a variety of individual factors. For example, urinary total NNAL increases with the number of cigarettes smoked per day[43,44] and might depend on age, gender, and race.[44,59] Furthermore, the intensity of smoking, which also affects constituent exposures in smokers, is influenced by the rate of nicotine metabolism: clearance of nicotine is faster in active metabolizers, which leads to higher intensity of smoking in an effort to support a desired level of nicotine (56). Although

based on the results of prior research[10,11,43,45,60] we expect that dose will be the major determinant of exposure to TSNA, examination of individual factors such as demographics, smoking history and intensity, and nicotine metabolism may provide an important information about the effect of these factors on the relationship between the machine-measured TSNA per mg nicotine yields in cigarette smoke and biomarkers of exposure in smokers. Genetic differences in nicotine and TSNA metabolism can affect intensity of smoking and resulting exposure to these constituents.[61,62] However, the effect of genotype on the relationship between smoke yields and biomarker levels is beyond the scope of this proposal.

Relevance to the research priorities identified by the FDA

FDA identified seven research areas in which additional scientific evidence is needed for the regulation of tobacco product manufacturing and marketing. One of such areas is "Reducing toxicity and carcinogenicity of tobacco products and smoke".[63] Our proposal directly addresses two research priority questions listed in this area: "*What in vitro and in vivo assays are capable of comparative toxicity between two different tobacco products; with special attention to cardiotoxicity, respiratory toxicity, carcinogenicity, and developmental/reproductive toxicity?*" and "*How should the impact of reduced levels of harmful and potentially harmful constituents of tobacco products on toxicity and carcinogenicity be measured*?" In addition, the proposed approach to the measurement and interpretation of the levels of tobacco carcinogens and their biomarkers can be used to understand differences in carcinogen exposures from tobacco use other than cigarette smoking, for example novel smokeless tobacco products. Therefore, this study might also provide important information for a priority question listed under the "Understanding the diversity of tobacco products" research area: "*What biomarkers of exposure should be used to measure exposure to new and emerging tobacco products?*"

Summary

Scientific evidence supports the important role of tobacco and cigarette smoke carcinogens in the development of cancers associated with cigarette smoking. Regulation of the levels of harmful constituents in cigarette smoke is one of the tobacco control strategies that now can be employed by the FDA and may serve to reduce tobacco carcinogen exposures in those smokers who are unable or unwilling to quit smoking. Such regulation will require a valid and robust approach to the assessment of comparative toxicity among various cigarette brands. Analysis of smoking machine-measured constituent yields per mg nicotine is a promising alternative to the conventional testing approaches, which are based on 'per cigarette' emissions. However, there is no evidence that constituent per mg nicotine yields are related to constituent exposure. We will address this research gap by investigating the relationship between the machine-measured NNN and NNK per mg nicotine yields in smoke of U.S. cigarettes and the biomarkers of exposure to these carcinogens in the urine of smokers who smoke these cigarettes. We will also investigate which of the commonly used smoking machine regimens produces NNN and NNK per mg nicotine yields that are stronger correlated with the uptake of these carcinogens in smokers, and will explore whether the 'TSNA per nicotine' content in the spent cigarette filters can be used as a proxy for this uptake. A secondary aim is to explore individual factors that may moderate the relationship between constituent yields and biomarkers of exposure. Other ancillary objectives will be also pursued to generate valuable information (relationship between TSNA and PAH yields in smoke) or resources (sidestream cigarette smoke, biological samples collected from study subjects) for future studies.

B. Innovation

Although the TobReg Study Group has previously proposed the measurement of cigarette smoke constituents per mg nicotine as a tool for the assessment of cigarette smoke toxicity, the group also suggested to limit the goal of these measurements to product performance evaluation, shifting away from the attempts to estimate actual exposures in smokers. However, tobacco product regulation by the FDA cannot be separated from its effects on tobacco users. Therefore, it is imperative to understand how this approach to the interpretation of the machine-measured cigarette smoke emissions is related to the exposures in smokers. The innovation of the proposed research consists in employing our group's unique combination of expertise in tobacco product analysis, biomarker measurements, and clinical trials to investigate this relationship. There are several novel elements in this study. First, we will fill the information gap on the TSNA per mg nicotine emissions in the currently marketed U.S. cigarette brands, and on the effect of different smoking machine regimens on these emissions. Additionally, we will explore the relationship between TSNA and PAH yields in the U.S. cigarettes, and this information is of great value for tobacco regulatory science. Second, previous studies that examined TSNA exposures in U.S. smokers either featured a brand-switching design (smokers of regular cigarettes temporarily switching to reduced-TSNA cigarettes) or compared urinary NNAL levels between smokers of

regular and "light" cigarettes which do not necessarily differ in NNK per mg nicotine content. This study will be first to investigate how customary smoking of cigarettes that differ in TSNA yields per mg nicotine affects the uptake of these carcinogens in U.S. smokers. Third, we will explore a novel approach to the assessment of TSNA per mg nicotine intake in smokers by analyzing mouth-level exposure to these carcinogens based on TSNA:nicotine analysis in cigarette filters. This approach might be extremely useful as an effective non-invasive tool in the future assessments of the effect of changes in the machine-measured constituent per nicotine yields in cigarette smoke on the smokers' exposures. There are very few studies besides the tobacco industry research that employed cigarette filter analysis. Finally, we will explore whether certain individual factors affect the relationship between the machine-measured TSNA per mg nicotine yields and exposures in smokers and therefore should be considered when examining constituent/mg nicotine smoke yields for regulatory purposes.

C. APPROACH

Overview

This project includes two major phases: analysis of constituent yields in the smoke of U.S. cigarettes (Aim 1) and analysis of biomarkers of exposure in smokers (Aim 2). Quantitative relationships between the measurements carried out in these two phases will be analyzed in Aim 2, and individual factors that may moderate these relationships will be explored in the secondary Aim 3.

Specific Aim 1. To examine NNN and NNK per mg nicotine emissions in the smoke of various U.S. cigarette brands under different smoking machine regimens.

In this specific aim, we will purchase several popular cigarette brands and will smoke these cigarettes on a smoking machine at three different conditions: FTC/ISO (non-intense), Massachusetts (medium), and Canadian (intense). According to its draft guidance for tobacco industry, the FDA recommends measurement of constituent amounts in cigarette smoke by two methods, non-intense and intense, but does not specify exact smoking regimens to be used.[3] Therefore, we will use three different smoking conditions that are commonly used by the industry in reporting smoke constituent yields.

We have two primary goals in this Aim. First is to analyze the yields of NNN and NNK per mg nicotine in the mainstream smoke. Based on the available data in the literature, and on our own preliminary experiments and calculations, we hypothesize that (i) NNN and NNK per mg nicotine yields will vary at least 3-fold among the U.S. cigarette brands, and (ii) that the non-intense FTC/ISO regimen will produce lower TSNA 'per cigarette' yields but higher TSNA 'per nicotine' yields than the intense Canadian regimen. Second is to measure nicotine, NNN, and NNK levels in the spent filters of the machine-smoked cigarettes. This information is important to Aims 2 and 3 of this proposal. In Aim 2, we will explore whether spent cigarette filters from our study subjects can be used as a proxy of their NNN and NNK per mg nicotine intake. Therefore, it is important to understand whether or not the 'per nicotine' amounts of NNN and NNK trapped in the spent filter are representative of the levels of these constituents in the smoke. In Aim 3, we will use the absolute 'per filter' nicotine levels in the spent cigarette filters as a measure of smoking intensity. Therefore, the amounts of nicotine in the filters of machine-smoked cigarettes will serve as a reference in the evaluation of smoking intensity in smokers.

Our ancillary objective in this aim is to examine the relationship between the TSNA and PAH yields in the smoke of U.S. cigarettes. While our preliminary data indicate that there is no significant reverse relationship between these groups of constituents in the smoke of a limited set of U.S. cigarettes, it not known whether this lack of relationship will sustain for a more diverse set of cigarette brands.

The proposed research will be accomplished in the following way:

Selection of cigarette brands and styles. Whereas the number of cigarette brands marketed in the U.S. is not extremely large, there are numerous styles or varieties for each brand. For example, according to the Philip Morris website, there are 63 styles of Marlboro cigarettes, and according to the RJ Reynolds website there are 25 styles of the Camel brand. The various styles differ in cigarette structure (length, filter ventilation), blending of tobacco types, as well as the presence or absence of menthol; these differences can significantly affect the total, or 'per cigarette', yields of constituents in the smoke of these cigarettes. However, it is not known how these features may affect the constituent per nicotine yields.

Based on the available preliminary data on the variation in TSNA per mg nicotine yields in the same U.S. cigarettes smoked by three smoking regimens (Table 2), we will need 99 cigarette samples to assure statistical significance of the differences among the three regimens (please see "Sample size" below). For each major

brand, we will make an effort to have a good representation of diverse styles to include different cigarette sizes, pack colors (for example, "gold" or "blue", which may be equivalents of former "lights" and "milds"). Estimated numbers of styles that may be analyzed for various brands are shown in Table 4. Our previous study on TSNA levels in U.S. cigarettes included 8 out of 11 brands listed in Table 4.[8] Thus, we expect to find at least 3-fold difference in TSNA yields among the brands and styles that will be analyzed in this study.

Table 4. Cigarette brands and estimated numbers of styles to be analyzed in Aim 1

Brand	Number of styles to be included in analysis[a] / total styles available[b]	% Smokers in Minnesota[c] / nationwide[d]
Marlboro	35 / 63	31.7 / 39.9
Camel	15 / 25	11.4 / 6.4
Newport	2 / 2	10.1 / 9.9
Kool	4 / 7	4.1 / 1.3
Winston	4 / 6	3.6 / 2.8
Salem	4 /7	2.7 / 1.5
Pall Mall	6 / 12	2.6 / 5.7
Doral	6 / 10	2.4 / 2.6
Basic	10 / 23	2.4 / 3.8
Merit	8 / 15	2.3 / 0.7
Parliament	6 / 10	2.1 / 1.5

[a] Styles will be selected to include different cigarette sizes, pack colors, and mentholated versions.
[b] According to manufacturers' websites.
[c] Pooled data from studies conducted at the Tobacco Research Programs, University of Minnesota.[64]
[d] Based on market share data reported by various sources.[65-67]

Smoking regimens and cigarette smoke collection. We will use a single-port CSM-SCSM smoking machine from CH Technologies (Westwood, NJ), which is compatible with any length and diameter of cigarette and is equipped with a software allowing to program specific smoking regimens. Each cigarette brand will be smoked at three conditions (ISO/FTC, Massachusetts, and Canadian intense) as described in the "Methods and procedures" section, and the mainstream smoke will be collected on Cambridge filter pads. Spent cigarette filters from each smoked cigarette will be placed in an individual container. The machine also allows for simultaneous collection of sidestream smoke in a separate trapping device. While the analysis of sidestream smoke is beyond the scope of our project, we will collect this fraction for future analyses. It was shown that relative amounts of various cigarette smoke constituents differ between mainstream and sidestream smoke. Moreover, we observed that the ratio of total NNAL to cotinine is different in nonsmokers exposed to secondhand smoke than in smokers.[68] Therefore, analysis of sidestream smoke from our cigarettes might be of interest for the tobacco regulatory science that deals with secondhand smoke exposures in nonsmokers.

Analysis of cigarette smoke and spent filters. We possess extensive experience and all the necessary equipment to conduct analyses proposed here. In our previous work, we predominantly used gas chromatography (GC) coupled with the nitrosamine-selective Thermal Energy Analyzer (TEA) for TSNA analysis,[69,70] however in our recent analysis of cigarette smoke samples we applied liquid chromatography (LC)-tandem mass-spectrometry (MS/MS).[8] We also routinely analyze nicotine by GC-MS.[70] In this study, we will use LC-MS/MS instrumentation. This will not only minimize sample preparation steps, but will also allow to simultaneously extract and analyze nicotine and TSNA in the same smoked Cambridge pad or cigarette filter.[71] We also developed an analytical procedure for the simultaneous determination of at least 23 different PAH in a single tobacco sample.[22] We will apply this methodology to analyze eight carcinogenic PAH in the smoked pads: benzo[*a*]pyrene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, indeno[1,2,3-*cd*]pyrene, dibenz[*a,h*]anthracene.[22] Thus, by using our two robust procedures, we will be able to generate data on multiple cigarette smoke constituents. For quality control purposes, we will include reference cigarettes 1R3F, 2R4F, and 1R5F (College of Agriculture Reference Cigarette Program at the University of Kentucky) with each set of cigarettes passing through the smoking machine.

Statistical analyses. Repeated measures analysis of variance will be utilized to analyze the data. We will compare 3 smoking regimens, testing each style of cigarette under each regimen and making triplicate measurements of each cigarette. Styles will be nested within brands. Regimen and brand will be crossed. We will use F tests to determine whether the NNN and NNK per mg nicotine yields vary among the U.S. cigarette brands and construct contrasts between brands to estimate the fold differences. In addition we will construct contrasts between the regimens and determine whether the non-intense FTC/ISO regimen will produce lower TSNA 'per cigarette' yields but higher TSNA 'per nicotine' yields than the more intense regimens. Pearson's correlations will be used to test the relationship between NNN and NNK 'per nicotine' levels in the spent filters of machine-smoked cigarettes and NNN and NNK 'per nicotine' yields in the smoke.

Sample size. In order to achieve 90% power to detect a difference of -0.318 (calculated based on available data of delivery per mg nicotine reported for a limited number of U.S. cigarettes smoked under various smoking regimens) between the null hypothesis correlation of 0.0 and the alternative hypothesis correlation of 0.318, we

require a sample size of 99 cigarettes, using a two-sided hypothesis test at a significance level of 0.05. Thus, we choose to sample 100 cigarettes, from approximately 10-11 brands.

Potential problems, alternative strategies, and benchmarks for success. Due to our broad experience with the analysis of tobacco products, we are well prepared to deal the typical problems that arise while carrying out analytical chemistry protocols: occasional issues with scheduling the use of laboratory equipment or mass-spectrometry instruments, temporary lack of supplies, occasional poor chromatography, low performance of mass-spectrometers that can be resolved by their cleaning and tuning, and other problems with procedures. The project Principal Investigator, Dr. Irina Stepanov, has an extensive firsthand experience in the analysis of TSNA and other toxicants in cigarettes, smokeless tobacco, and nicotine replacement therapy products.[69,72-75] In addition, we enjoy the support and commitment of Dr. Clifford Watson, who is an expert in smoking machine regimens and cigarette smoke collection (please see enclosed letter of support). He will advise us on these procedures should any problems arise, which further assures a successful implementation of Aim 1. The major outcome of this Aim that will mean its successful implementation is to have nicotine, NNN, NNK, and PAH content in 100 samples of U.S. cigarettes measured by three smoking machine methods that differ in intensity.

Specific Aim 2. To examine the extent to which differences in smoke yields of NNN and NNK per mg nicotine are predictive of differences in smokers' exposure to these carcinogens.

In Specific Aim 2, we will recruit habitual smokers of cigarette brands that deliver three different levels of NNN and NNK per mg nicotine in the smoke (as established in Aim 1), 100 smokers per group. We will measure total nicotine equivalents, total NNN, and total NNAL in the urine of the recruited smokers. Spent cigarette filters will be also collected from each smoker and analyzed for nicotine, NNN, and NNK.

We have two primary goals in this Aim. First is to compare urinary total NNN and total NNAL (with and without normalization for total nicotine intake) among the three groups of smokers and with the machine-measured yields of NNN and NNK per mg nicotine. Based on the reported studies and our preliminary data, we expect that the levels of urinary total NNN and total NNAL normalized per total nicotine equivalents will be different among the groups and will correlate with cigarette smoke yields. Even though, based on the data in the literature, we expect that urinary biomarkers will not correlate with 'per cigarette' yields of NNN and NNK, we will analyze this relationship for reference purposes. We will also examine whether one of the smoking machine regimens used to measure smoke levels of NNN and NNK per mg nicotine in Aim 1 better correlates with total NNN and total NNAL per nicotine equivalents in smokers' urine. Second goal is to compare, for each smoker, the TSNA per mg nicotine content in their spent cigarette filters to the levels of total NNN and total NNAL per nicotine equivalents in their urine. We expect that spent filter measurements will strongly correlate with the levels of urinary biomarkers.

This study will be carried out in the following way:

Selection of cigarette brands for the targeted recruitment of smokers. Based on the results of the measurements in Specific Aim 1, we will identify three sets of popular cigarette brands that differ in NNN and NNK per mg nicotine yields in smoke. NNN and NNK both are formed via nitrosation of tobacco alkaloids, and this process is affected by similar factors. Therefore, for any two cigarette brands, differences in NNN yields in the smoke are generally accompanied by the comparable differences in NNK content. This represents an important advantage for the proposed study, allowing for simultaneous investigation of the relationship between the smoke yields and biomarker levels for both carcinogens.

Table 5. Differences in 'per mg nicotine' yields among U.S. cigarette brands[a]

Cigarette brand[b]	Emissions in cigarette smoke, ng/mg nicotine	
	NNN	NNK
Group 1		
Camel	100.1	56.3
Pall Mall	103.9	66.2
Kool	113.1	52.5
Group 2		
Marlboro	155.5	82.1
Winston	172.8	78.5
Group 3		
Basic	207.1	146.1
Doral	282.4	125.5

[a] Yields are calculated based on our data for NNN and NNK measured by the FTC/ISO method [8] and available nicotine values from the FTC report.[48]
[b] Full flavor, king size cigarettes in hard packs.

Based on our analyses of NNN and NNK and the reported nicotine levels the last FTC report on "tar" and nicotine levels in cigarette smoke,[48] we estimate that NNN and NNK per mg nicotine yields in the smoke of U.S. cigarettes vary at least 3-fold, and that certain popular brands can be assembled in three distinct groups that differ in these yields (Table 5). Analysis of a larger set of brands, as proposed in Aim 1, will allow us to establish ranges for the three levels of NNN and NNK per mg nicotine yields to be targeted in Aim 2, and to select a representative set of brands and

styles for each level. Based on the preliminary calculations summarized in Table 5, we expect to be able to assemble cigarette brands in three groups so that that there is ~50% increase in the average NNN and NNK per mg nicotine yields in group 2 compared to group 1, and the same increase in group 3 compared to group 2. We are aware of the reported ~11% increase in 'per cigarette' nicotine yields since the last FTC report.[76] However, this change is not expected to alter the degree of variations in TSNA per mg nicotine yields among the three levels.

Subject recruitment and sample collection. We will recruit adult daily smokers, making an effort to include a wide spectrum of age, socioeconomic status, race/ethnicity, and light and heavy smoking. The inclusion criteria are listed in the "Methods and procedures" section following the overview of specific aims. Smokers meeting these criteria and invited to the clinic for an orientation visit will be asked to bring their regular cigarettes for brand identification, to avoid uncertainties related to the self-reported variety/style of cigarettes. We will collect 24-h urine samples and spent filters from all cigarettes smoked the day of urine collection so that a representative value for each measurement can be obtained. These measurements will be carried out at a single clinic visit because previous study that analyzed urinary total NNAL in samples collected over 3-5 consecutive days from smokers who smoked constant number of cigarettes per day showed little variation of this biomarker within the same subject.[77] Moreover, a longitudinal study that measured total NNAL in smokers' urine sampled bimonthly for 1 year demonstrated that single total NNAL measurements are reliable indicators of the typical levels over that 1-year period, and that the measurements within an individual vary much less than they do from person to person.[78] Given that total nicotine equivalents account for the majority of nicotine metabolites, this biomarker is an excellent and stable indicator of the total nicotine dose. We will also collect a separate spot urine sample at the time of clinic visit to test whether a single urine sample can be used instead of 24-h collections in future studies.

There also could be batch-to-batch variations in smoke composition within the same cigarette brand. Therefore, we will provide each subject with their cigarette brand to smoke over one week prior to their clinic visit, and will ask them to return all unsmoked cigarettes when they come to the clinic. A subset of the returned cigarette samples will be selected from each group to test whether TSNA/mg nicotine yields are in agreement with those established in Aim 1, and to compare these yields to urinary total NNN and total NNAL per total nicotine equivalents in smokers who smoked these cigarettes at the time of urine collection.

In addition to urine samples, we will also collect blood samples and buccal cells for potential future analyses of tobacco biomarkers or genotyping for tobacco carcinogen metabolizing genes. These measurements may become important for the analyses proposed in Aim 3, or other studies of tobacco constituent exposure and metabolism. Also, in the future, samples may be analyzed for genetic predisposition for tobacco toxicant metabolism, behavior and harm.

Analysis of urine and spent cigarette filters. The collected 24-h and single spot urine samples will be aliquoted into 5-mL cryovials for biomarker analyses, and each vial will be labeled with a unique bar code which will encode the study, subject, and the type of assay. The aliquots will be frozen at -20 ºC until analyses for total nicotine equivalents, total NNN, total NNAL, and creatinine. We will also save additional aliquots for potential future analyses of other tobacco-related biomarkers that may become important or may be developed over time. While we will collect all spent cigarette filters from the day prior to clinic visit, we will analyze only 3 randomly selected filters for each study subject, to obtain an average level of the mouth-level NNN and NNK per mg nicotine exposure at the day of urine collection. Urine samples and cigarette filters will be analyzed by methods described later in the section "Methods and procedures".

Statistical analyses. A one-way ANOVA will be performed to check for differences between the means of the biomarkers by the 'per mg nicotine' yield groups 1, 2, and 3 (see Table 5), assuming the variances are similar. P-values from further pair-wise comparisons will be adjusted by the Bonferroni method. If the variances are not similar, two-sample t-tests will be performed using p-values of 0.0166 as statistically significant. If normality does not hold, a log transformation on the raw data or the non-parametric Kruskal-Wallis test will be considered. Correlations between the continuous measures of machine measured yields of NNN and NNK per mg nicotine and total NNN and total NNAL will also be evaluated. Parametric and non-parametric (Spearman and/or Kendall) correlation coefficients and multiple regression analysis will be used to evaluate the correlation between these measures adjusting for gender, age, race and smoking history and intensity. Correlations between the machine measured yields of NNN and NNK per mg nicotine and total NNN and total NNAL will be estimated within each of the 3 smoking machine regimens. Parametric and non-parametric (Spearman and/or

Kendall) correlation coefficients and multiple regression analysis will be used to evaluate the correlation between these measures. 95% confidence intervals will be given.

To appropriately account for the within-subject correlation of multiple filters per subject, a linear mixed model will be employed to measure the association between the TSNA:nicotine content in the filters and the endpoints of urinary biomarkers.

Sample size. Based on the available data for NNAL/Cotinine ($x10^3$) (see Table 3), we calculated the power to detect a 50% increase in from group 1 to group 2, to group 3. With a sample size of 100 smokers per group, and assuming that we are using 3 two-sided two-sample tests with a significance level of 0.0166 (=0.05/3) for each comparison, we will achieve 96.4% power between groups 1 and 2, 98.6% power between groups 2 and 3, and >99% power between groups 1 and 3.

Potential problems, alternative strategies, and benchmarks for success. Generally, we do not expect significant complications in this Aim. The differences in TSNA per mg nicotine emissions have been demonstrated for a limited set of the U.S. cigarettes, and we expect that these differences will persist in a larger selection of cigarette brands. The methodology for biomarker analyses is well established and validated, and the project PI Dr. Irina Stepanov has an extensive experience in the development and application of biomarkers of tobacco carcinogen exposure.[79-83] Therefore, she is well qualified to deal with the usual challenges related to analysis of large numbers of samples for trace biomarkers. In addition, Dr. Sharon Murphy, who is an expert in nicotine metabolism and is routinely measuring nicotine biomarkers in her laboratory, will consult us on the analysis of total nicotine equivalents. There may be concerns about subject recruitment. However, Dr. Dorothy Hatsukami, project Co-Investigator, has years of experience conducting clinical trials that tested tobacco products for patterns of use and toxicant exposure.[11,45,84-90] Drs. Stepanov and Hatsukami also have a track record of successful collaborations that combined their expertise in clinical trials and biochemical analyses.[82,83,91,92] There could be also concern about the ability to detect differences in NNN and NNK per mg nicotine exposure across different cigarette brands. However, our calculations based on the available preliminary data indicate that our sample size is large enough to detect these differences. We also have on board Robin Bliss, who is a senior clinical trial biostatistician and has been collaborating with Drs. Stepanov and Hatsukami on a number of tobacco research projects.[93-96] The major outcome of this Aim that will mean its successful implementation is to measure biomarkers of exposure to nicotine, NNN, and NNK in the urine of 300 smokers who smoke selected cigarette brands, and to measure nicotine, NNN and NNK in smoked cigarette filters of these study subjects.

Specific Aim 3. To determine which individual factors may affect the relationship between the machine-measured TSNA per mg nicotine and exposures in smokers.

In this secondary Aim, we will investigate how such factors as demographics, duration and intensity of smoking, and nicotine metabolism affect the relationship among the measurements conducted in Aims 1 and 2. Examining these factors is important in order to understand which aspects will need to be taken into consideration when examining constituent/mg nicotine smoke yields for regulatory purposes.

Data on demographics and smoking history will be collected at the orientation visit. To evaluate the potential effect of smoking intensity on the relationship between NNN and NNK per mg nicotine in smoke and biomarker levels in smokers, we will use the amount of nicotine measured in spent cigarette filters. The ratio of nicotine levels in spent filters collected from smokers to the levels measured in filters from the same cigarettes smoked by smoking machine operating in the least intense FTC/ISO regime will be used as an indicator of smoking intensity. The effect of nicotine metabolism will be assessed via the ratio between two nicotine metabolites measured in Aim 2, cotinine and trans-3'-hydroxycontinine (3-HC). This ratio is used as an indicator of the enzymatic activity of CYP2A6 – the primary enzyme associated with nicotine metabolism.[97,98]

The effect of genotype on the relationship between smoke yields and biomarker levels is beyond the scope of this proposal. However, blood and oral cell samples collected in Aim 2 may be used as DNA source for potential future genotyping for nicotine and TSNA metabolizing genes.[61,62]

Statistical analyses. We will utilize regression to model the effects of demographics, duration and intensity of smoking, nicotine metabolism, and NNN and NNK per mg nicotine in smoke, on biomarker levels in smokers. The analysis will start with a graphical exploration of the associations between NNN and NNK per mg nicotine with biomarker levels. Assuming the relationship is linear, added variable plots and partial residual plots will be utilized to determine whether adding demographics, duration and intensity of smoking and nicotine metabolism, affects the relationship between NNN and NNK per mg nicotine with biomarker levels. A regression approach with a forward selection procedure will be used, such that at each step the variable with

the largest F statistic is added to the model, after including either NNN or NNK per mg nicotine. Model diagnostics will be conducted, i.e. looking for nonconstant variance, nonlinearity, collinearity, assumptions of normality. Transformations will be considered. The significance level for entry into the model will be 0.05.

Sample size. With a sample size of 100 we achieve 95% power to detect an R-Squared of 0.13 attributable to either NNN or NNK per mg nicotine using an F-test at a significance level of 0.025. If NNN or NNK per mg nicotine accounts for 20% of the variance in biomarker levels, then with a sample size of 100 we achieve 95% power to detect an additional R-Squared of 0.16 due to 6 variables such as gender, age, smoking duration in years, number of cigarettes smoked per day, smoking intensity, and nicotine metabolism when using an F-test at a significance level of 0.025.

Potential problems, alternative strategies, and benchmarks for success. This Aim is using data generated in Aims 1 and 2. Therefore, success of this aim to a large degree depends on overcoming any potential complications in Aims 1 and 2. One concern relevant to this aim may be the representativeness of the smoker population, so that gender, age, race, and smoking history and intensity are adequately diverse. Therefore, we will make sure that the sample has good representation of these factors. This is an exploratory aim, and therefore the benchmarks for its success cannot be clearly identified. The major outcome of this aim will consist in determining whether or not there is a significant moderating effect of the studied individual factors on the relationship between NNN and NNK per mg nicotine in cigarette smoke and the relevant biomarkers in smokers' urine.

Methods and procedures

Cigarette analyses

Cigarette acquisition. The cigarettes will be purchased from retail stores in Minnesota, each pack will be assigned a unique identification number, and the details regarding cigarette brand, style, and other characteristics, along with the information on the date and place of purchase will be entered in a database. According to the FDA draft guidance, tobacco manufacturers are expected to provide constituent data for 7 replicates (20 replicates for nicotine and CO) and to provide information that is "representative to the product as marketed".[3] For the purpose of this proposal, we will purchase 3 packs of cigarettes per brand/style to obtain triplicate measurements per sample. For each brand/style, three packs will be purchased in three different retail stores to have a reasonably representative sample.

Smoking machine regimens. We will use three smoking regimens: (a) FTC method: cigarettes are smoked by drawing 35 mL puff volumes over 2 s, with 60 s interval between puffs and no blocking of filter ventilation holes; (b) the more intense Massachusetts method: 45-mL puffs drawn over 2 s, with 30 s interval between puffs and 50% blocking of filter ventilation holes,[37] and (c) Health Canada intense smoking regimen: 55-mL puff volumes of 2-s duration, with 30 s interval between puffs and 100% blocking of filter ventilation holes.[38] For each individual sample, two cigarettes will be smoked per condition, and the mainstream smoke will be collected on Cambridge filter pads (1 cigarette per pad) pretreated with ascorbic acid to prevent artefact formation of TSNA.

Analysis of smoke constituents. One of the two Cambridge filter pads obtained for each cigarette sample and regimen will be used for nicotine, NNN, and NNK analysis, and the second one will be used for PAH analysis. For nicotine, NNN, and NNK analysis, filter pads will be spiked with deuterium-labeled NNN, NNK, and nicotine (internal standards), extracted with ammonium acetate solution, and analyzed by LC-MS/MS as described.[8,10,99] For PAH analysis, filter pads will be spiked with a mix of ^{13}C-labeled various PAH (internal standards) extracted with cyclohexane, and purified and analyzed by GC-MS/MS as previously described.[22,100] The GC-MS/MS method for PAH analysis allows to measure a total of 23 various PAH, including BaP.

Analysis of cigarette filters. One-cm portions will be removed from the mouth end of the spent cigarette filters, stripped of the wrapping paper and transferred into clean vials. Nicotine, NNN, and NNK will be extracted with ammonium acetate solution and analyzed as described.[10]

Human subject procedures

Recruitment. We will recruit 300 adult smokers from Minneapolis-St. Paul metro area. The subjects will be recruited through newspaper advertisements and initially screened over the telephone to determine if they are in good physical and mental health, have the required smoking history, and smoke one of the targeted cigarette brands. Smokers meeting these criteria will be invited to the clinic for an orientation visit.

Orientation visit. The subjects will learn about the details of the study, and sign a consent form and complete questionnaires on demographics, smoking, nicotine dependence, and health history. Subjects will be included if they are: 1) daily current smokers; 2) smoke at least 10 cigarettes per day; 3) smoked the reported brand/style for >80% of their cigarettes over the course of at least 1 year, and smoked this brand exclusively for at least two weeks prior to the eligibility screening; 4) not using any other nicotine or tobacco product; 5) in good mental and physical health (as determined by the medical history and PRIME-MD, a psychiatric screening tool,[101] 6) not taking medications that might affect the metabolism of nicotine or TSNA (for each subject, inquiries will be made regarding medication use and investigators will determine if these medications might influence nicotine or TSNA); and 7) not pregnant (determined by pregnancy test) or nursing women. Eligible subjects will be scheduled for the next clinic visit (scheduled for the following week). They will be provided with their brand of cigarettes to smoke over one week prior to the clinic visit (the number of cigarette packs will be estimated based on their self-reported cigarettes per day). The subjects will be given containers for urine and cigarette filter collection, and asked to collect a 24-h urine sample the day prior to their scheduled clinic visit, and to bring the urine sample and all spent filters from cigarettes smoked over that day to the clinic. Subjects will be also asked to bring all the remaining unsmoked cigarettes back to the clinic.

Clinic visits. At the clinic visit, the 24-h urine sample, spent cigarette filters, and the remaining cigarettes will be collected. A spot urine sample will be collected to compare biomarker ratios in this sample with those measured in the 24-h urine sample collected over the previous day. Vitals will be taken, exhaled CO will be measured, and blood and oral cell samples will be collected.

Collection of blood. Blood samples will be collected into 'purple top' blood collection tubes, and plasma, red blood cells, and buffy coat will be separated by centrifugation and stored at -80 ºC for future studies.

Collection of oral cells. Buccal cells will be collected by scraping inside the mouth with a cytobrush. Two cytobrushes will be used on each subject. Cells collected on one cytobrush will be used for DNA isolation with the Puregene Buccal Cells Kit. These samples will be available for future genotyping. Cells collected with the second cytobrush will be stored in RNAprotect saliva reagent so that RNA can be isolated in the future and used for gene expression analyses. Oral samples will be stored at -80 ºC.

Carbon monoxide (CO) test. The measurements will be made using the Bedfont Micro Smokerlyzer® (Bedfont Scientific Limited, Kent, UK) measurement device.

Compensation. Each subject will be paid $50 for their efforts.

Biomarker analyses

Urinary carcinogen biomarkers. Total NNN and total NNAL will be analyzed by the standard validated methods used in our laboratory.[82,83,102,103] Briefly, urine samples are mixed with stable isotope-labeled internal standards ([pyridine-D_4]NNN or [$^{13}C_6$]NNAL) and treated with β-glucuronidase to release free NNN and NNAL from their glucuronides. The samples are further purified using solid-phase extraction cartridges. The appropriate eluants are then analyzed by LC-MS/MS monitoring transitions m/z 178 → 148 for NNN, m/z 184 → 154 for [$^{13}C_6$]NNN, m/z 210 → 93 NNAL and m/z 216 → 98 for [$^{13}C_6$]NNAL.

Total nicotine equivalents. Analysis of these biomarkers will be conducted by gas chromatography-mass spectrometry after treating urine with ß-glucuronidase to cleave the glucuronide conjugates, as previously described.[104-106]

Rate of nicotine metabolism. This rate will be assessed via the ratio of 3-HC to cotinine, which reflects the enzymatic activity of CYP2A6 and reflects the extent of nicotine clearance rate.[97,107]

Hazardous materials

TSNA and some PAH are potent carcinogens and will be handled with extreme care, in a well-ventilated hood and with personal protective equipment. The study staff involved in collection and analysis of urine and mouthwash samples will have completed the necessary safety and bloodborne pathogen training, and the required immunizations.

PROTECTION OF HUMAN SUBJECTS

Risks to Human Subjects

Human Subjects Involvement, Characteristics, and Design. Human subjects are essential to this study as their enrollment will allow to investigate exposure resulting from smoking various cigarette brands. This exposure is one of important endpoints in tobacco product toxicity testing, and complements the analyses of constituents in cigarette smoke.

A total of 300 male and female smokers of specific cigarette brands (the brands established in Aim 1) will be enrolled over the course of 3.5 years. The enrollment will begin after the completion of the initial phase of the study, which is cigarette smoke analysis. We anticipate enrolling at least 8 subjects a month. Enrolling this number of subjects is not unreasonable: we have experience of enrolling and randomizing over 400 subjects in one year.

Timeline:

Period	Year 1	Year 2	Year 3	Year 4	Year 5
Study preparation and Initiation	2 mos				
Aim 1 – cigarette analysis	10 mos	6 mos			
Aim 2 – subjects enrolled		N=85	N=85	N=85	N=45
Final data analysis and manuscript submissions					6 mos

The inclusion criteria for will be the following:

a) Male or female adult smokers 18-65 years of age, who normally smoke at least 10 cigarettes per day (to assure that biomarker levels are above the limit of quantitation);
b) Smoked the same brand for >80% of their cigarettes over the course of at least 1 year, and smoked this brand exclusively for at least two weeks prior to the eligibility screening;
c) Not using any other nicotine or tobacco product;
d) Subjects are in good physical health (no unstable medical condition) and good mental health (e.g. not currently, within the past 6 months, experiencing unstable or untreated psychiatric diagnosis, including substance abuse, as determined by the PRIME-MD);
e) Subjects who are not taking any medications that affect relevant metabolic enzymes;
f) Women who are not pregnant or nursing;
g) Subjects have provided written informed consent to participate in the study (adolescents under the age of 18 will be excluded because this project involves use of tobacco products).

The exclusion criteria are the following:

a) Significant immune system disorders, respiratory diseases, kidney or liver diseases or any other medical disorders that may affect biomarker data;
b) Current or recent alcohol or drug abuse problems (to ensure alcohol and drug use does not affect biomarkers of exposure and to maximize retention);
c) Regular tobacco use (e.g., greater than weekly) other than cigarettes;
d) Currently using nicotine replacement or other tobacco cessation products (to minimize confounding effects of another product);
e) Pregnant or breastfeeding (due to toxic effects from tobacco products).

Based on our prior studies, we anticipate that we will recruit subjects between the ages of 18 to 70, with the mean age at around 40 years old, slightly more males than females (40% females), 30% minority groups and of middle to lower socio-economic status.

Subjects will be recruited through various media (internet, television, newspaper, radio). The advertisements would read as follows: *Daily smokers needed for a study testing cigarettes that contain different amounts of harmful tobacco chemicals. Smokers will not be asked to change their usual brand of cigarettes. We want to know if different cigarette brands change exposure of smokers to these chemicals*. Cigarette smokers will contact our clinic and be screened for eligibility over the telephone. During this screening, information will be obtained on where the subject heard about the study, their geographic location, and basic demographics. This

data will provide information on the primary media avenue, radio or television station, advertisement placements, internet location or domain responsible for recruiting subjects. We don't anticipate difficulty in recruiting a significant minority population based on our prior experience. However, if we find that the recruitment levels fall below 30%, we will advertise in community newspapers, post in public places (e.g., stores, restaurants and bars, laundry facilities, churches and community centers).

We will maximize retention of the subjects between the orientation and clinic visit by compensating them for their time.

Sources of Materials. At orientation, subjects will be asked to complete questionnaires on medical history, demographics and tobacco use. During the study, subjects will be required to continue smoking their usual cigarettes at usual rate. Subjects will be required to collect a 24 h urine sample and save all smoked cigarette butts the day prior to their clinic visit. During the clinic visit, we will assess for blood pressure, heart rate, and breath samples for alveolar CO will be taken. A capillary blood sample and oral cell samples will also be collected. Information about each subject will be entered into a database by the Study Coordinator. Each subject will be coded with a unique number, and only these coded ID's will be entered into the database. All raw data will be kept in locked file cabinets.

Only the Study Coordinator and Co-Principal Investigators will have access to individually identifiable private information about subjects. Coded ID's will be used throughout the study by all the researchers involved. While all the samples and information will be collected specifically to achieve the goals of this proposal, de-identified individual subject data and back-up samples may be available to other researchers for research purposes after our study is complete. A separate consent form will be used to obtain permission from subjects to allow de-identified biosamples to be stored in a biorepository for future analyses of biomarkers or genotyping.

Potential Risks. The potential risks for subjects recruited for this study are minimal. We will provide subjects with their regular cigarette brands and will ask them to smoke cigarettes at their regular rate. We will provide them with no more cigarettes than self-reported rates. The cigarettes will be purchased at retail stores and only unopened packs will be dispensed to study subjects. Physiological and subjective measures will be noninvasive and should present no psychological or medical risk to the subject. Blood samples and oral samples will be obtained by a registered nurse or trained technician. Subjects will be under medical supervision throughout their study participation and adverse events from study procedures will be monitored by the project PI Dr. Stepanov and Co-I Dr. Hatsukami.

At the end of the trial, subjects will be strongly encouraged to stop use of all tobacco products and to set a quit date, and provided with a treatment resources and referral to different treatments including the state quit line.

Adequacy of Protection Against Risks

Recruitment and Informed Consent. Potential subjects will be told the nature of the research over the phone during screening and then at an orientation meeting. They will be told they may discontinue participation at any time and will not be discriminated against if they choose to do so. Subjects will be told their participation in the project will be strictly confidential, that any identifying information will be available to the site investigators only, and that no identifying information concerning the data and results will be made known. Subjects will have written assurance that while de-identified individual subject data may be available to other researchers for research purposes, only a summary of the results will ever be published or otherwise publicly released. They will also be informed that all raw data will be coded with numbers and kept in locked file cabinets. Subjects will be required to demonstrate an understanding of the study purpose and procedures prior to signing the consent form. Consent form must be signed before the research is started.

The subjects will be told they may discontinue participation at any time and will not be discriminated against if they choose to do so.

Protections Against Risk. All data will be de-identified and posted on a secure, password-protected website that is only available to the investigators in this project. All identifying information will be in a locked cabinet in a secure place. Subjects will be screened for any potentially compromising medical condition and will be monitored throughout the study. Subjects will be encouraged to quit smoking at the end of study.

Potential Benefits of the Proposed Research to Human Subjects and Others

Whereas no assurance can be made to an individual subject that he/she will personally benefit from the research, the experience should be beneficial. Subjects will have the opportunity to learn about factors that may be associated with smoking. Quitting smoking will be strongly recommended to our subjects and cessation materials will be provided. Referrals to community resources will also be made.

The risks in relation to potential benefits are minimal to the individual research subject and virtually nonexistent to society in general.

Importance of the Knowledge to be Gained

This study will result in recommendations for methods and measures for the evaluation of cigarette smoke toxicity. Tobacco use causes about one third of all cancer deaths. While majority of current smokers in the U.S. are interested in quitting, tobacco use is highly addictive. Those smokers who are unable or unwilling to quit, are being exposed to unduly high levels of certain human carcinogens. The knowledge gained in this study could be used to reduce the levels of known human carcinogens in the currently marketed cigarette brands and to prevent the entry into the market of new cigarettes that may result in even greater harm than the existing brands.

Data and Safety Monitoring Plan

Daily oversight of subject safety will be conducted by the study coordinator and the research nurse. The Principal Investigator Irina Stepanov and Co-Investigator Dorothy Hatsukami will meet weekly with the study staff to review recruitment progress and any adverse events. Entrance criteria will be reviewed following screening. Since we will be providing subjects with cigarettes, medical history will be reviewed for any contraindications for the continuation of smoking. Vital signs will be checked at orientation and at the clinic visit. Smokers will be under medical supervision while in the study and our research staff will make appropriate referrals to the physician should any adverse events occur.

In addition, a Data and Safety Monitoring Board, which will be comprised of three experts in the areas of smoking tobacco products, biomarkers and clinical trials, will be convened. The Board will be comprised of individuals internal and external to the University with expertise in tobacco and tobacco-related toxicity, and will include individuals with an MD, a PharmD and a PhD and a statistician. They will begin by reviewing the protocol and establishing guidelines for data and safety monitoring. This will include developing standard procedures for day-to-day monitoring by the internal reviewers and study staff. This Board will meet at regular intervals to evaluate the progress of the trial, review data quality, patient recruitment, study retention, and examine other factors which may affect study outcome. They will also review the rates and types of adverse events to determine whether there has been any change in participant risk. Their review will ensure that subject risk does not outweigh the study benefits. A brief report will be generated from each of these meetings for the study record and forwarded to the University of Minnesota's Institutional Review Boards (IRB).

All adverse events from study procedures of a non-serious nature will be reported to the University of Minnesota's IRB at the time of renewal. Serious adverse events from study procedures will be reported by telephone to the IRB within 3 days of our receipt of information regarding the event and written reports will be submitted within 10 days. The Data and Safety Monitoring Board will review all serious or unexpected adverse events and provide recommendations.

We will inform NIH of any significant action taken as a result of the Data and Monitoring Board's findings.

INCLUSION OF WOMEN AND MINORITIES

We will recruit subjects from the local metropolitan area by advertisements placed in campus and metropolitan newspapers, radio or television. The metropolitan area is sufficiently large in population to ensure an adequate sample of subjects with diverse demographic and ethnic backgrounds. Men and women of all races and ethnicity will be recruited for the study. In the 2011 census, the average percentage of minorities across the Hennepin county, MN was 22.8% of the population (Black = 12.0%, Asian = 6.5%, American Indian = 1.2%, other = 3.1%). The Hispanic population is 6.9%. We will make special efforts to solicit participation of minorities and women by advertising in community newspapers, local church organizations, and community centers. We will recruit women who are neither pregnant nor nursing.

Targeted/Planned Enrollment Table

See link below for updated notice:

http://grants.nih.gov/grants/guide/notice-files/NOT-OD-14-086.html

INCLUSION OF CHILDREN

Because tobacco products are illegal for use among minors, we will be excluding children and adolescents from the proposed studies. Participants will be restricted to adults over the age of 18 who can consent to be in the studies. We will however recruit children between the ages of 18 and 21 years.

RESOURCE SHARING PLAN(S)

A Data Sharing Plan will be established to facilitate the sharing and analysis of data with the research community. Data generated by this grant will be made available to outside investigators, according to the Guidance at http://grants.nih.gov/grants/policy/data_sharing/data_sharing_guidance.htm. Data will be available in two formats: as summary graphs and tables, and as raw data files for analysis. However, this will not be available until papers are accepted for publication. When data are shared, there will be no limits placed on how the data will be used, and co-authorship is not required as a condition for receiving data. Users will agree, however, that the recipient not transfer the data to other users and that the data are only to be used for research purposes. A record of transfer of data and a copy of the dataset that was distributed will be kept by the project PI.