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EFFECT OF MENTHOL FLAVOR IN CIGARETTES ON ADDICTION AND TOXICANT EXPOSURE

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ABSTRACT

The United States Food and Drug Administration (FDA) is considering two tobacco product standards in cigarettes including banning menthol and reducing nicotine content. In April 2022, FDA proposed product standards to prohibit menthol as a characterizing flavor in cigarettes. The overall goal of this dissertation is to provide novel information on whether menthol in cigarettes modifies the addiction potential in cigarettes and exposure to toxic constituents. While the health effects of menthol have been studies for decades, there remains unanswered questions on aspects of its effects. These include whether menthol affects exposure to tobacco smoke carcinogens and nicotine metabolism. Menthol cigarette smoking may impact nicotine metabolite ratio (NMR) and facilitate exposure to tobacco smoke. Few studies have examined the differences in urine NMR and biomarkers of tobacco smoke between menthol and non-menthol cigarette smokers. We used data from the National Health and Nutrition Examination Survey (NHANES) 2015-2016 special sample to compute urine NMR by menthol status. NHANES 2015-2016 special sample data were also used to compare markers of FDA's 26 Harmful and Potentially Harmful Constituents (HPHCs) in tobacco smoke. These markers include urinary metabolites of polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs) and metals. Urinary metabolites of 7 PAHs, 15 VOCs and 4 metal biomarkers were compared between exclusive menthol (n=162) and non-menthol (n=189) cigarette smokers. Multivariable analysis was conducted on creatinine-adjusted concentrations. We found that adjusted geometric means (GMs) of urine NMR were 1.8 and 1.6 for menthol and non-menthol cigarette smokers, respectively. There was no difference in NMR by cigarette flavor (Ratio of GMs [Menthol/Non-menthol]: 1.1; 95% CI: 0.8,1.6; p>0.1). Menthol did not affect the nicotine metabolite ratio in urine. There were no significant differences in cotinine levels and in 22 of the HPHCs. Among the metabolites of PAHs, the levels of 1-hydroxyphenanthrene were

about 16% lower in menthol smokers. Among the metabolites of VOCs, menthol cigarette smokers presented significantly lower concentrations of acrylamide (115.6 µg/g creatinine vs.154.6 µg/g creatinine; Ratio of GMs [95% CI]: 0.7 [0.6,0.9]; p-value: 0.02), N, N-dimethylformamide and acrylonitrile. Menthol and non-menthol smokers presented similar levels of metals.

In addition to menthol flavor, nicotine reduction is another possible strategy considered by FDA to decrease prevalence of cigarette smoking. In 2018, the U.S. FDA issued an Advanced Notice of Proposed Rulemaking (ANPRM) to reduce nicotine in tobacco products to make them minimally addictive or nonaddictive. The present dissertation evaluated whether menthol modifies the treatment effect of a gradual reduction from fully nicotinized cigarettes to very low nicotine cigarette (VLNC 0.4mg/cig) over a 5-month period of time. Two parallel randomized clinical trials of gradually reduced nicotine in cigarettes from 11.6 mg down to 0.2 mg nicotine (very low nicotine content; VLNC) over an 18-week period in smokers with low socioeconomic status (SES) and mental health conditions. Menthol did not modify the pooled association between nicotine content treatment (VLNC vs. UNC [Usual Nicotine Content]) and cigarettes per day, expired carbon monoxide levels, nicotine dependence and symptomology. Menthol did not have a differential impact on multiple subjective ratings of VLNC cigarettes, except harshness. The odds ratio for being compliant for non-menthol vs. menthol users is 2.6 (95% CI:1.0, 6.4; p-value: 0.04).

In summary, the impact of menthol on nicotine metabolism is not significant between menthol and non-menthol cigarette smokers. Menthol and non-menthol cigarettes deliver similar levels of HPHC. Findings on toxicity are similar for menthol and non-menthol cigarettes. The pooled analysis of clinical trials indicated that lowering nicotine content in cigarettes had similar beneficial effects by flavor status, except menthol smokers were less likely to smoke VLNC

cigarettes without supplementation with usual cigarettes. These findings provide support for a menthol ban if nicotine content in cigarettes is reduced to very low levels. The information from these studies should be informative to the FDA on whether to implement a final ruling on a ban and respond to potential challenges on any proposed ban.

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Chapter 1

Introduction and Literature Review

1.1 Epidemiology of Tobacco Use

The prevalence of cigarette smoking has decreased from 20.9% (2005) to 14.0% (2019),[1] but cigarette smoking is still the primary preventable cause of death in the United States (U.S.) and is responsible for more than 480,000 deaths annually.[2] More specifically, cigarette smoking causes one in five deaths annually, or 1,300 deaths every day.[2]

There are more than 7,000 chemical compounds in cigarette smoke.[2] Some of them are well-known human carcinogens and others are toxic agents that contribute to smoking-related illness such as heart disease and lung disease.[2] Due to the carcinogens contained in the cigarettes, smoking causes many types of cancers, including stomach cancer, bladder cancer, and almost 90% of lung cancer cases.[2] In addition to the cancers mentioned above, new diseases were linked to smoking and secondhand smoke, for example, diabetes, colorectal cancer, liver cancer, and tuberculosis according to the 2014 Surgeon General's Report.[2] More than 16 million people in the U.S. live with a disease caused by smoking [2] and smokers' life expectancy was shortened by more than 10 years compared to non-smokers.[3]

Besides, the economic burden of smoking is staggering; the total economic cost of smoking is estimated at more than $300 billion a year, approximately $170 billion is spent for direct medical care [4] and at least $156 billion is incurred in lost productivity as a result of premature death and secondhand smoke exposure.[2]

Cigarettes, hookah, and cigars are usually classified as combustible products, whereas chew, dissolvable, electronic cigarette, and snuff are classified as non-combustible products.[5] Although cigarettes are the most commonly used tobacco products in the US, the prevalence of other tobacco product use, for instance, e-cigarette has increased rapidly since it entered market in 2007.[6] The increased prevalence of ever use of e-cigarettes is a new public concern.[7] The popularity of e-cigarettes might be explained by the perception of cheaper price and more socially acceptable than other traditional combustible tobacco products.[8] Additionally, e-cigarettes do not contain most of the harmful constituents in tobacco and tobacco smoke [9] may further contribute to current situation. Some prior research recommend that e-cigarettes have potential in harm reduction and can replace conventional cigarettes,[10,11] whereas some systematic reviews cast doubt on the use of e-cigarette in cessation aid and risk reduction.[12,13] Thus, the evidence is mixed and the long-term health risks of e-cigarettes are not yet well established.[7]

Menthol cigarette use

Menthol, a monocyclic terpene alcohol, is widely used by tobacco companies as a characterizing flavor in cigarettes.[14] Menthol can be produced from peppermint plant or the corn mint. It can also be synthetically made.[14] Menthol plays an important role in combining certain neuronal receptors that regulate pain, taste and other sensation of brain, which contributes to its unique proprieties in cooling.[14]

Many tobacco experts have criticized the use of menthol as an additive in cigarettes because it hides the unpleasant harshness or bitter taste of cigarette smoke and aversive physiological effects of smoking.[15,16] Due to its “masking” function, smoking menthol-flavored cigarettes may increase smokers’ exposure to nicotine, [17-19] the likelihood of smoking initiation especially among youth.[15,20-24] According to the study that analyzed from the Population

Assessment of Tobacco and Health (PATH) 2013-2014 data, almost 60 % of youth used menthol cigarettes, whereas less than 38% of adults used menthol-flavored cigarettes.[25] In addition to smoking initiation, a few cross-sectional and longitudinal studies recommended that compared to non-menthol cigarette users, young menthol cigarette users were more likely to become established cigarette users ("established smoking" is usually defined as " at least 100 cigarettes lifetime plus smoking on 20–30 of the past 30 days"). [24,26-28]Besides, menthol is responsible for prolonged elevation of blood cotinine levels [29,30] as well as intensifying the nicotine dependence by activating certain brain regions.[31,32] Further, the prior study conducted by Míguez-Burbano et al. suggested that menthol cigarette users displayed increased risk of hypertension (almost twice compared to non-menthol smokers), higher body mass index, and abdominal obesity among people living with HIV.[33]

There is a long history of concerns about menthol in cancer control research and prevention. In 2002, there was the National Conference on research needs regarding menthol cigarettes. To protect public and create a healthier future for all Americans, Congress passed the Tobacco Control Act in June 2009.[34,35] The 2009 Family Smoking Prevention and Tobacco Control Act authorized the FDA to ban all characterizing flavors in cigarettes except for menthol. [35,36] The 2nd National Conference on menthol in 2009, 4 months after the Act was passed, the 2011 Tobacco Products Scientific Advisory Committee (TPSAC) report to the FDA that recommended that "removal of menthol cigarettes would benefit public health in the United States".[14] In 2018, the U.S. FDA issued an Advanced Notice of Proposed Rulemaking (ANPRM) to reduce nicotine in tobacco products to make them minimally addictive or nonaddictive.[37] In April 2022, FDA proposed product standards to prohibit menthol as a characterizing flavor in cigarettes.

1.2 Nicotine metabolite ratio

Multiple prior studies indicate menthol cigarette flavor prevents the metabolism of nicotine in liver [38] and further affects the clearance of nicotine.[29] The enzyme cytochrome P4502A6 (CYP2A6) is the most important enzyme that metabolizes nicotine in liver.[39] Approximately 70% of nicotine will be metabolized into cotinine due to the enzyme activity of CYP2A6.[40] Next, more than half of cotinine will be further metabolized to trans-3'-hydroxycotinine (3HC).[41] The nicotine metabolite ratio (NMR), referred to as the ratio of 3HC to cotinine (3'hydroxycotinine [3HC]/cotinine), is a well-established index of CYP2A6 enzyme activity and it is widely used as a measure of nicotine metabolic activity.[42]

Higher NMR has been found to be associated with higher daily cigarette consumption and less success stopping smoking in cessation trials.[43] A few studies suggested that smokers with lower NMRs had better success at quitting than smokers with higher NMRs.[44,45] Lerman et al. found that smokers with higher NMRs were significantly less likely to remain abstinent.[45] However, the longitudinal study conducted by Fix et al. found that smokers with higher NMRs might be less likely to relapse following a quit attempt.[43] Their findings differed from a few existing studies, where smokers with lower NMRs had been found to be more likely to maintain abstinence from smoking.

1.3 Menthol interactions with nicotine

The research conducted by Fagan et al. found that young adult daily menthol smokers presented significantly lower nicotine metabolite ratio (NMR) compared to non-menthol smokers (0.19 vs. 0.24 p=0.03).[30] Another study conducted by Chenoweth et al. also indicated that menthol cigarette smokers displayed a significantly lower NMR than non-menthol cigarette users

(0.32 vs. 0.37, $p<0.001$).[46] Moreover, different studies find that menthol inhibits CYP2A6 activity in vitro[38] and nicotine clearance in vivo,[29] which may explain the low NMR observed from clinical studies. However, there is an ongoing debate regarding the menthol effect on NMR. For instance, Ho et al. found that menthol cigarettes did not significantly impact the nicotine NMR after adjusting for age, gender, and body mass index (BMI).[47] Another case-control study also revealed that there was no difference in NMR between menthol and non-menthol smokers after controlling for race.[48]

The ultimate goal of tobacco control is cessation.[49] NMR has been considered as a reliable predictor of smoking cessation outcomes in a few clinical studies.[45] Instead of using the same and general treatment for all smokers, the personalized cessation pharmacotherapies based on smokers' NMR has been found successful with higher efficiency [50,51] and lower cost.[50] Specifically, nicotine patch is suitable for smokers with slow NMR, whereas varenicline (a medicine works by blocking the pleasant effects of nicotine from smoking on the brain) is recommended for smoker with fast NMR.[47,50,51] These individualized cessation treatments usually lead to maximum treatment response and minimum side effects of cessation aid.[50] Therefore, the tailored treatment based on smokers' NMR can bring a significant clinical benefits by optimizing quit rates and minimizing side effects at the same time.[50]

1.4 Biomarkers of tobacco exposure

As mentioned previously, there are more than 7,000 chemical compounds in mainstream of cigarette smoke. Some of them are well-known human carcinogens and the rest of them are toxic agents, including ammonia, acrolein, acetone, carbon monoxide, benzopyrenes, hydroquinone and nitrogen oxides.[52]

Menthol flavor may also play an important role in impacting biomarkers of tobacco exposure. A few studies had been conducted to compare the biomarkers of tobacco smoke by menthol status. Most of them focused on nicotine metabolites, carbon monoxide (CO), and tobacco-specific nitrosamines. [48,53-59]Some of the studies indicated that higher levels of cotinine and CO among menthol cigarette users compared to non-menthol smokers,[48,53,57-59] but some of them concluded that the difference between them in serum cotinine were not statistically significant. In addition to the biomarkers listed above, other tobacco-related biomarkers such as blood cadmium and lead were also investigated. Jones et al. found that menthol cigarette users presented higher levels of blood cadmium (Ratio of geometric mean; Menthol/Non-menthol: 1.10 (95% CI: 1.04-1.16)) compared to non-menthol smokers, but not with other biomarkers (lead and NNAL).[60] Cadmium, a toxic and carcinogenic tobacco constituent,[61] is responsible for cancers of lung and prostate.[62] Further, it also contributes to increased risk of cardiovascular disease, kidney disease, and bone disease.[61,63-68] As for the major source of cadmium, the soil that used to grow tobacco plants usually contain cadmium, and the amount of cadmium in cigarette is increased during the tobacco production process.[69,70]

1.5 National Health and Nutrition Examination Survey

The National Health and Nutrition Examination Survey (NHANES) aims to assess the health and nutrition status of adults and children in the United States. NHANES is a publicly available dataset with a stratified, multi-stage, random probability sampling design conducted by the National Center for Health Statistics (NCHS) at the Center for Disease Control and Prevention (CDC) in the United States.[71] Further, NHANES provides a nationally representative sample of the civilian, noninstitutionalized U.S. population. [71]

The NHANES program began in 1960s and investigated a few series of surveys targeting at different study populations and public health topics. There are demographic, socioeconomic, dietary, and health-related questions in the NHANES interview.[71] In addition, well-trained medical personnel will obtain medical, dental, physiological, and laboratory tests information from participants.[71]

The NHANES has been one of the most important national datasets which provides valuable health and nutritional status of U.S. adults and children. For instance, findings from this national survey can help with determine the prevalence of major disease and risk factors, which can be used by public health facilities and hospitals for allocation of health care resources as well as developing public health policy and directing services.[71]

The NHANES has consistently provided analytes of tobacco exposure biomarkers since its inception and in the special sample, data on urinary biomarker concentrations became available in 2015-2016. The primary objective of this dissertation is to examine NMR and biomarkers of tobacco exposure between menthol and non-menthol smokers using 2015-2016 special sampling waves. The NHANES 2015-2016 obtained data from both smokers and nonsmokers. Participants aged 18 years and older, from regular one-third subsample, were included in the special subsample.[71] Additionally, to oversample adult smokers, those participants aged 18 years and older, not in the regular one-third subsample, who smoked at least 100 cigarettes in their entire lifetime and now smoke cigarettes every day, were also included in the special subsample. Adult smokers were defined as participants who had smoked at least 100 cigarettes lifetime and now smoke cigarettes every day (Figure **1-1**).[71,72]

The NHANES laboratory data included multiple biomarkers of tobacco exposure, such as cotinine, total urinary (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol) (NNAL), and polycyclic aromatic hydrocarbons (PAHs). Cotinine is the main metabolite of nicotine, and it is available in blood, urine, and saliva.[73-76] NNAL is a carcinogen formed from NNK (tobacco-specific n-

nitrosamines).[77-80] PAHs are usually generated by incomplete combustion of tobacco and other organic components during smoking.[81] For example, benzo[a]pyrene (BaP) is classified as Group 1 carcinogen, one of the most potent carcinogens.[82]

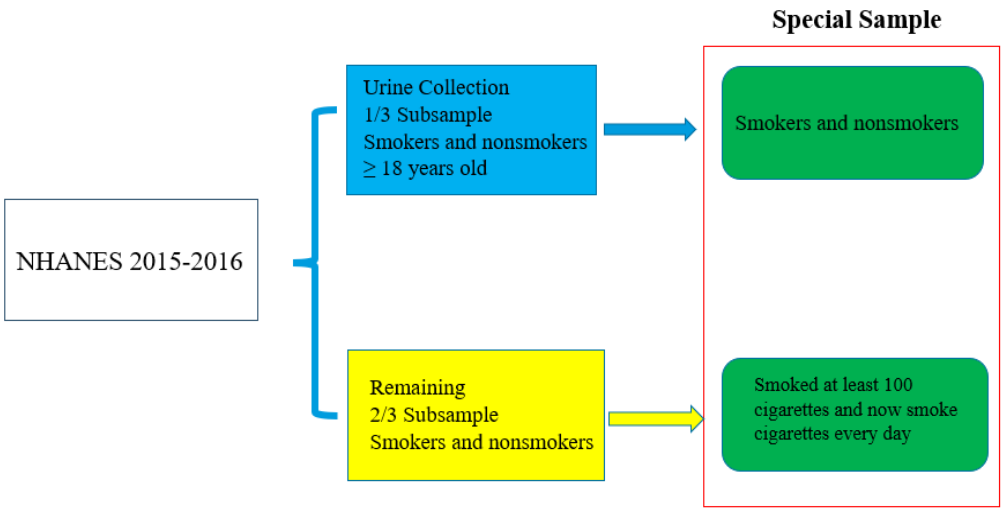

Figure **1-1**. NHANES 2015-2016 Special Sample

Analysis of biomarkers (NHANES)

Urinary cotinine, trans-3'-hydroxycotinine, urinary metabolites of PAHs, volatile organic compounds (VOCs) and metals are the main biomarkers of tobacco exposure available in the NHANES Special smoker sample. Urine specimens are processed, stored, and shipped to the Division of Laboratory Sciences, National Center for Environmental Health, and Centers for Disease Control and Prevention for analysis.[71] Vials are stored under frozen (–30°C) conditions until they are shipped to National Center for Environmental Health for testing.[71] The lower limit of detection (LLOD in ng/ml) for cotinine, trans-3'-hydroxycotinine, OH-PAHs was listed in Table **1-1**.[71] An imputed fill value was used in the result section if analytes had a concentration below the lower limit of detection. NHANES sets the imputed value equal to the lower limit of detection divided by the square root of 2 (LLOD/sqrt[2]).[71]

Table **1-1.** Lower limit of detection (LLOD) for biomarkers of tobacco exposure [71]

Analyte Name	LLOD
Total Cotinine, urine (ng/ml)	0.03
Total Hydroxycotinine, urine (ng/mL)	0.03
Nicotine, urine (ng/mL)	10.5
1-Hydroxynapthalene (1-Naphthol) (ng/L)	60
2-Hydroxynapthalene (2-Naphthol) (ng/L)	90
3-Hydroxyfluorene (ng/L)	8
2-Hydroxyfluorene (ng/L)	8
1-Hydroxyphenanthrene (ng/L)	9
1-Hydroxypyrene (ng/L)	70
2-Hydroxyphenanthrene & 3-Hydroxyphenanthrene (ng/L)	10

1.6 Reduced nicotine content cigarettes

Nicotine is the key component that plays a crucial role in addiction.[83] The Family Smoking Prevention and Tobacco Control Act enacted by U.S. Congress in 2009 granted the FDA the authority to regulate tobacco products to decrease the prevalence of cigarette use.[84] The FDA announced an Advanced Notice of Proposed Rulemaking (ANPRM) in 2018 aims for nicotine reduction.[85] The logic behind the nicotine reduction policy is to achieve considerably declined smoking prevalence by reducing nicotine dependence progression and stopping onset of smoking in new smokers.[86] Enactment of the nicotine reduction policy may bring a tremendous positive effect on public health. For example, more than 16 million persons will not initiate smoking by 2060 due to the implementation of the policy, and 8.5 million tobacco-related deaths can be avoided by 2100 in the United States.[87]

Currently, there is a strong body of evidence that indicates cigarettes with reduced nicotine content (RNC) have multiple benefits, including reduced nicotine exposure levels as well as fewer cigarettes smoked per day. [86,88-92] There are mainly two strategies (immediate and gradual reduction) to reduce nicotine content in cigarettes.[86] Both immediately and gradually RNC approaches resulted in decreases in biomarkers of smoke exposure.[86,90]

The prior study conducted by Hatsukami et al. reported that the immediate reduction in nicotine content to very low nicotine content (VLNC) cigarettes resulted in decreased nicotine dependence, more cigarette free days, and reduced biomarkers of smoke exposure across time compared to the control group (usual nicotine content cigarettes at 15.5 mg of nicotine per gram of tobacco cigarettes).[86] Specifically, significant lower levels of breath carbon monoxide (CO; $P < 0.0055$), urine 3-hydroxypropylmercapturic acid (3-HPMA, metabolite of acrolein; $P < 0.0055$), and urine phenanthrene tetraol (PheT, indicator of polycyclic aromatic hydrocarbons; $P < 0.0055$) were observed in VLNC group compared to the control group.[86]

Nonetheless, due to the substantial reduction in nicotine content, the immediate reduction approach led to more intense withdrawal symptoms and smokers were more likely to obtain nicotine from other sources to alleviate their cravings.[86] The immediate reduction approach has great potential in decreasing nicotine dependence. However, there are some limitations, such as high drop-out rate and low study completion rate.

In addition to the immediate reduction study mentioned above, another study targeted at low socioeconomic status smokers conducted in Pennsylvania and Washington D.C. reported that gradually reduced nicotine content to VLNC (nicotine content: 0.2 mg/cigarette) also led to significant reductions in cigarette per day (CPD) and exposure biomarkers.[90] For instance, significant lower levels of the tobacco carcinogen biomarker NNAL were observed in the gradually RNC group compared to the control group.[90] The gradual nicotine content reduction method progressively decreased the nicotine content from regular content to low nicotine content, and further declined to very low nicotine content. The gradual reduction method gives us more comprehensive information about the effects of different nicotine content on cigarette users' nicotine dependence and smoking behavior. More importantly, it helps FDA to find the precise nicotine content that has greatest reduction in nicotine dependence.

VLNC treatment effect on smokers by menthol status

A secondary analysis of a randomized trial conducted across 10 sites in the U. S. found that the magnitude of change in smoking (cigarettes per day), and toxicant exposure was significantly smaller among menthol smokers compared to non-menthol smokers.[93] Specifically, for smoking behavior, both menthol and non-menthol smokers presented decreased number of cigarettes smoked per day (CPD) between VLNC and control group (normal nicotine content),

but the degree of reduction was significantly lower among menthol smokers than non-menthol smokers (-6.4 vs. -9.3 for menthol and non-menthol respectively; p interaction=0.04).[93]

Results of this secondary analysis suggested the possible roles played by menthol flavor in diminishing the treatment effect of immediately reduced nicotine content (RNC) cigarettes on smoking behavior, biomarkers of toxicant exposure as well as cessation.

Two randomized clinical trials

Two randomized clinical trials were conducted by us to assess treatment effects of gradual reduced nicotine content cigarettes on adherence to study cigarettes and plasma cotinine concentration.

PSU-GWU was a two-arm, double-blind, parallel-group, randomized controlled trial. The primary endpoint of PSU-GWU trial was to determine adherence to study cigarettes with progressively reduced nicotine content.[94] The study population of PSU-GWU trial was cigarette smokers with low socioeconomic status (SES) population. Eligibility criteria included adult cigarette smokers aged between 18 to 65, smoked at least 5 cigarettes per day, and had no intention to quit in the next 6 months. SES trial (PSU-GWU) consisted of four phases, including usual brand baseline phase, usual nicotine content baseline phase, randomized phase, and treatment choice phase (SES trial, Figure **1**-**2**).[94] There were in total 11 clinical visits at the study centers where research staff collected demographic and biomarker information from participants.[94] The focus of this dissertation will be the randomization phase, where 245 participants randomly assigned to either Usual Nicotine Content (UNC) cigarettes group or progressively Reduced Nicotine Content (RNC) cigarettes group.[94] Participants in UNC group used 11.6mg/cigarette nicotine content cigarettes for 18 weeks, meanwhile nicotine content for RNC group gradually reduced every three weeks from 7.4 to 0.2 mg/cigarette (very low nicotine

content; VLNC).[94] During the randomization phase, participants in both groups attended in-person visits to complete different assessment forms including modified Cigarette Evaluation Questionnaire, and Cigarette Liking Scale. Research staff also collected blood samples for biomarkers analysis and investigation of study compliance.

Second trial was conducted at Penn State College of Medicine and Massachusetts General Hospital (PSU-MGH). PSU-MGH was also a two-site, two-arm, double-blind parallel-group, randomized clinical trial. The study protocol was similar to PSU-GWU trial as discussed above, 200 adult smokers were randomly assigned to either RNC or UNC group during 18-week randomization phase.[95] PSU-MGH trial targeted at smokers with the mental health (MH) conditions [mood and/or anxiety disorders] (MH trial, Figure **1-3**). Instead of investigating adherence to study cigarettes (SES trial), MH trial was interested in comparing plasma cotinine concentration between UNC and RNC groups.[95]

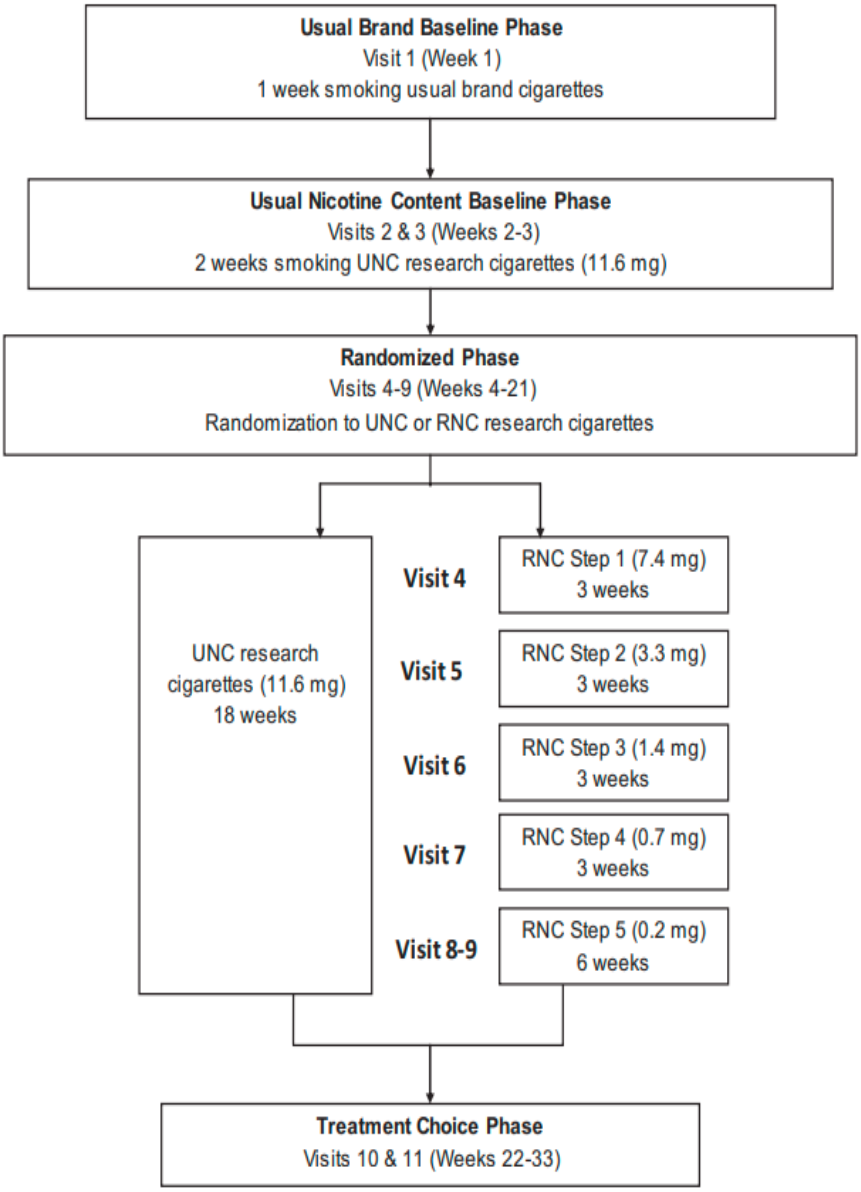

Figure **1-2**. Study flow diagram (SES trial)[94]

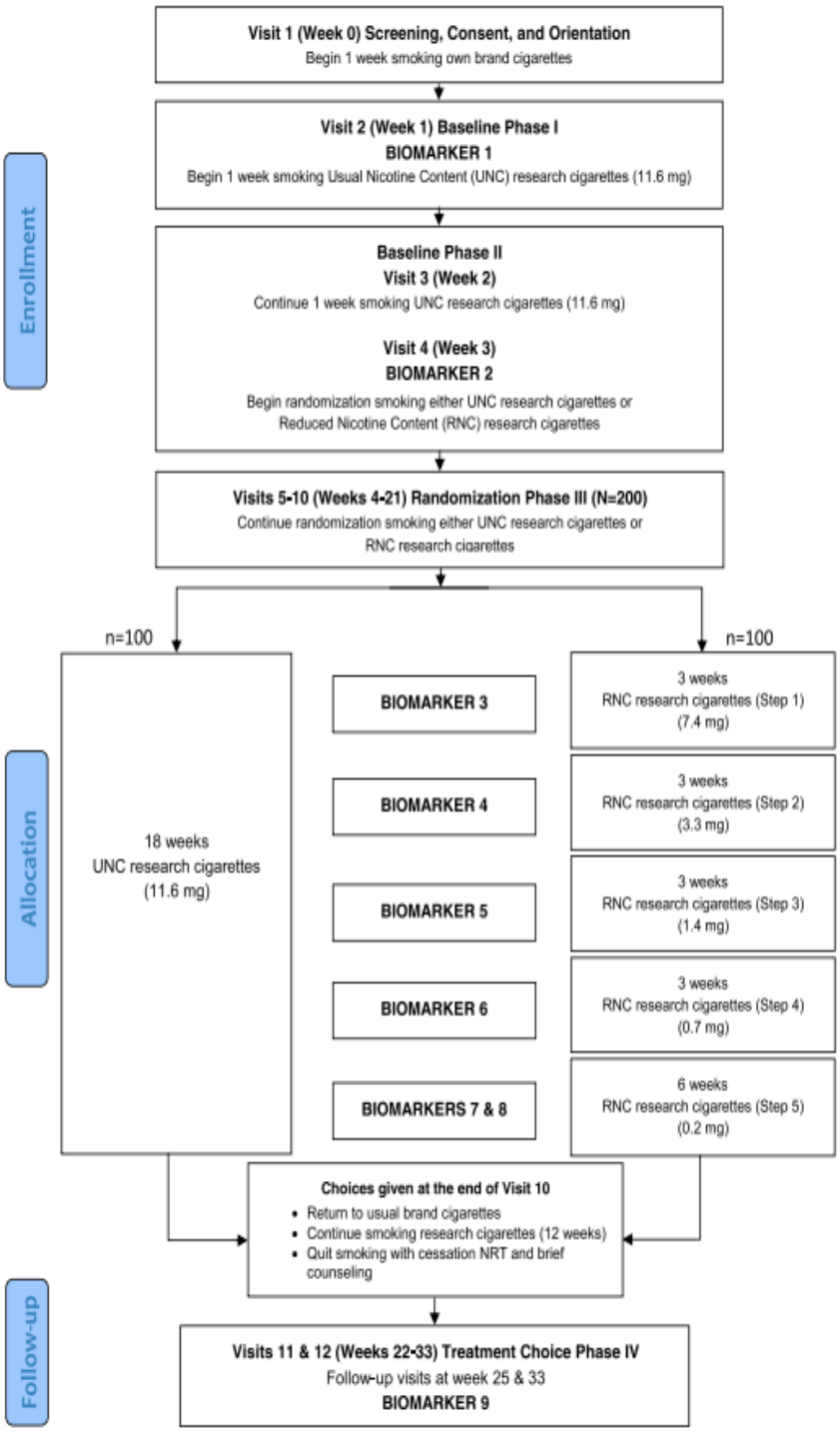

Figure **1-3.** Study flow diagram (MH trial)[95]

Subjective responses (modified Cigarette Evaluation Questionnaire, Cigarette Liking Scale)

Subjective responses included modified cigarette evaluation questionnaire (mCEQ)[96] and cigarette liking scale (CLS).[97] Table **1-2** listed 12 questions of mCEQ.[96] Specifically, item 1 ("satisfying"), item 2 ("taste good") and item 12("enjoy smoking") were regrouped to measure mCEQ subscale No. 1 ("Smoking Satisfaction"). Items 4 ("calm you down"),5 ("more awake"),6 ("less irritable"),7 ("concentrate"),8 ("reduce hunger for food") were regrouped to measure mCEQ subscale No. 2 ("Psychological Reward"). Items 9 ("dizzy") and 10 ("nauseous") were taken to measure "Aversion". Item 3 was taken to measure "Enjoyment of Respiratory Tract Sensations". Item 11 was used to measure "craving reduction". In addition to mCEQ, CLS was used to measure subjective responses of reduced nicotine content cigarettes (Table **1-3**) in a few studies.[97,98]

Subjective measures are important tools for measuring the acceptability and sensory effects of low nicotine products. Acceptability may modify the behavioral responses to reduced nicotine products used in trials, such as participant compliance and dropout. This could foreshadow the population-level response to a reduced nicotine policy. The study conducted by Smith et al. found that an immediate reduction in nicotine content led to a significantly lower product satisfaction score than a gradual reduction method ($p<0.001$).[99] The lower satisfaction score is related to greater reductions in smoking , smoke exposure, and nicotine dependence, which may further contribute to cessation.[99] However, due to the dramatic reduction in nicotine content, noncompliance has emerged as a potential concern.

In summary, the benefits of immediate VLNC include greater decrease in biomarkers of tobacco exposure and reductions in smoking due to the low satisfaction score. Nonetheless, it is also possible smokers may purchase normal nicotine content (NNC) cigarettes from "black

market" if NNC are no longer commercially available given the noncompliance observed from VLNC group with less satisfaction.[99]

As discussed previously, gradual reduction is another strategy considered by FDA in decreasing smoking prevalence. A few studies have investigated the potential benefits of gradual reduction and subjective response of gradual VLNC.[90,98] The prior study found that participants with reduced nicotine content (RNC) cigarettes had 50% lower (mean group difference −137 ng/mL; 95% CI −172, −102) in blood cotinine levels than the other group who used usual nicotine content (UNC) cigarettes.[90] Further, the RNC group also presented less cigarettes per day (CPD) (-4.1; 95%CI: -6.44, -1.75) and lower carbon monoxide levels (-4.0ppm; 95%CI: -7.7, -0.4) than UNC group.[98]

In addition to the treatment effect of gradual VLNC, subjective response of VLNC had been investigated. Specifically, visit 6 (nicotine content: 1.4 mg nicotine per cigarette) is the critical starting point of significant differences for both mCEQ and CLS measures between UNC and RNC groups.[98] Overall, RNC rated significantly lower score in satisfaction than UNC group beginning at visit 6 till the end of randomization phase at visit 9 (Figure **1-4**, Figure **1-5**, Figure 1-**6**, Figure **1-7**, and Figure **1-8**). But there was no difference between these two groups in rating aversion (negative reinforcements) during any visit.[98] Findings of subjective responses to gradual VLNC cigarettes highlight the important timepoint and the critical nicotine content that can be used by FDA in directing nicotine reduction policy for cigarettes.

Table **1-2.** Modified Cigarette Evaluation Questionnaire (mCEQ)[96]

If you have smoked since you last completed this questionnaire, please mark the number the best represents how smoking made you feel (1-not at all, 2-very little, 3-a little, 4-moderatly,5-a lot, 6- quite a lot, 7-extremely).	
1.	Was smoking satisfying?
2.	Did cigarettes taste good?
3.	Did you enjoy the sensations in your throat and chest?
4.	Did smoking calm you down?
5.	Did smoking make you feel more awake?
6.	Did smoking make you feel less irritable?
7.	Did smoking help you concentrate?
8.	Did smoking reduce your hunger for food?
9.	Did smoking make you dizzy?
10.	Did smoking make you nauseous?
11.	Did smoking immediately relieve your craving for a cigarette?
12.	Did you enjoy smoking?

Note: Items 1, 2, and 12 were taken to measure “Smoking Satisfaction”.

Items 4,5,6,7,8 were taken to measure “Psychological Reward”.

Items 9 and 10 were taken to measure “Aversion”.

Item 3 was taken to measure “Enjoyment of Respiratory Tract Sensations”.

Item 11 was taken to measure “Craving Reduction”.

Table **1-3.** Cigarette Liking Scale[97]

Item Number	Description
1	How strong was the cigarette? (1=not at all, 10=extremely)
2	How hot was the cigarette? (1=not at all, 10=extremely)
3	How hard was it to draw? (1=not at all, 10=extremely)
4	How harsh was the cigarette? (1=not at all, 10=extremely)
5	How much taste did you get from the cigarette? (1=not at all, 10=extremely)
6	How satisfying was the cigarette? (1=not at all, 10=extremely)
7	How much tobacco vs. 'just air' did you get from the cigarette? (1=just air, 10=just tobacco)
8	What is the likelihood that you would buy cigarettes like these? (1=not at all, 10=extremely)
9	How much nicotine do you think these cigarettes gave you compared to your usual cigarettes? (1=much less, 5=much more)
10	How satisfying was the hit these cigarettes gave you compared to your usual cigarettes? (1=much less, 5=much more)

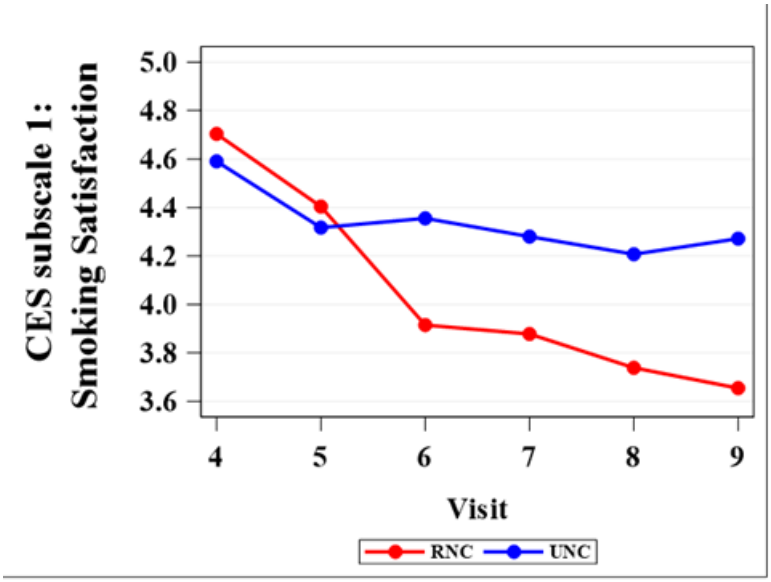

Figure **1-4.** CES subscale 1: Smoking Satisfaction

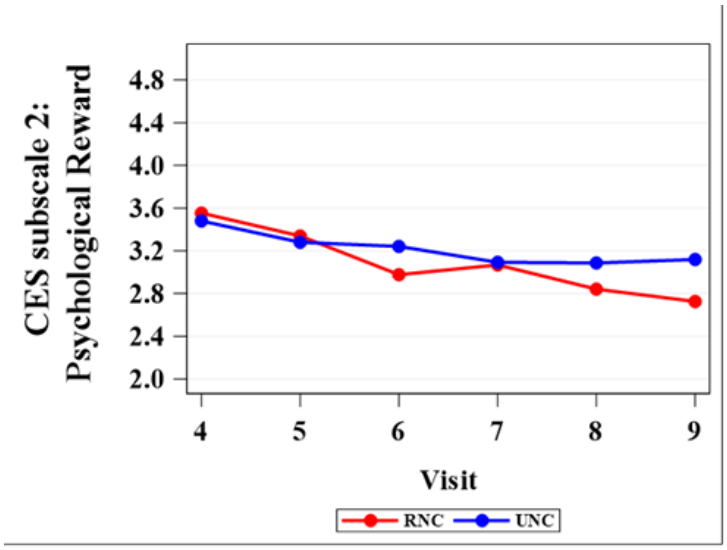

Figure **1-5.** CES subscale 2: Psychological Reward

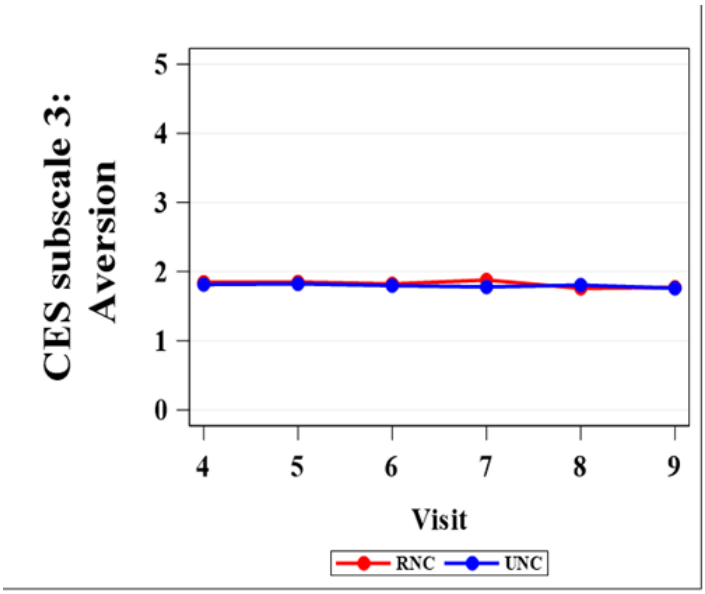

Figure **1-6.** CES subscale 3: Aversion

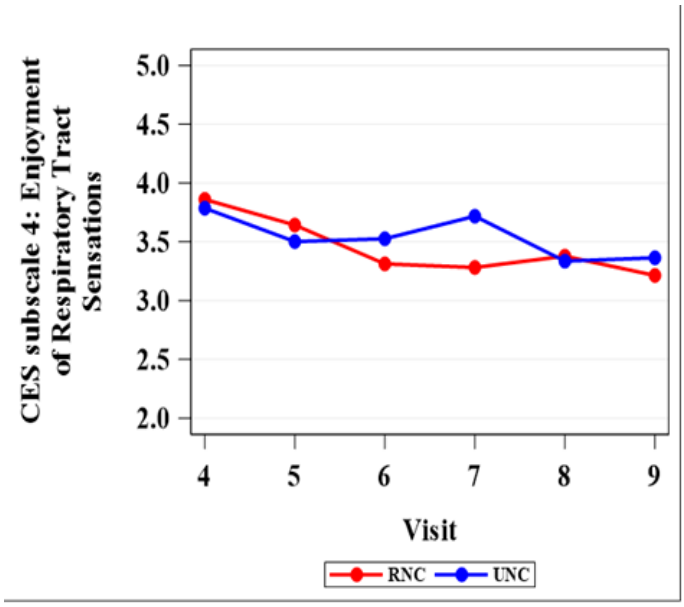

Figure **1-7.** CES subscale 4: Enjoyment of Respiratory Tract Sensations

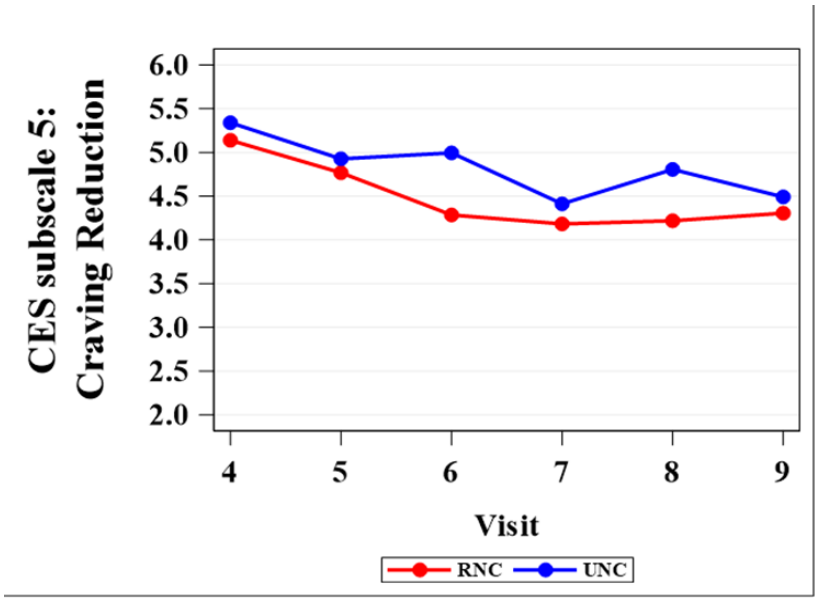

Figure **1-8.** CES subscale 5: Craving Reduction

Chapter 2

Research Gap and Purpose

2.1 Research Gap in the literature

A few epidemiologic studies investigated the potential roles played by menthol in NMR, but current research yielded conflicting results. Most studies used small sample size with limited generalizability, which may further contribute to inconclusive findings on the association between menthol flavor and NMR. Given NMR is critical for cessation treatment, it is necessary to conduct a comprehensive study to investigate the association between menthol and NMR with nationally representative sample of US smokers.

In addition to NMR, health impact of menthol flavor remains unclear. Some studies indicated the negative health impact of menthol on cigarette smokers, such as increased biomarkers of tobacco exposure, however, findings are inconsistent and controversial, which led to ongoing debate regarding the use of menthol as a cigarette flavor.

Menthol bans and reduced nicotine content cigarettes are two promising strategies considered by FDA in decreasing smoking prevalence. The prior research has recommended the menthol impact in diminishing treatment effects of immediate VLNC on biomarkers of tobacco exposure. Nonetheless, no epidemiologic study has investigated the menthol effect on gradual VLNC cigarettes. The relationship between menthol and gradual VLNC remains unknown, which needs attention from national and state health departments to answer this question.

Overall, this dissertation will fill in the research gap about menthol flavor by investigating the association between menthol, NMR, and biomarkers of tobacco exposure using the NHANES. Further, the randomized clinical trials will be used to provide comprehensive information about the roles played by menthol in VLNC treatment effect.

2.2 Specific Aims

Specific Aim 1: Examine the urine NMR between menthol and non-menthol cigarette users with nationally representative sample of US smokers.

Hypothesis: We hypothesize that the NMR is different between menthol and non-menthol cigarette smokers. We hypothesize a lower NMR in menthol smokers compared to non-menthol smokers.

Justification: The research conducted by Fagan et al. found that young adult daily menthol smokers presented a significantly lower nicotine metabolite ratio (NMR) than non-menthol smokers (0.19 vs. 0.24 p=0.03).[30] Another study conducted by Chenoweth et al. also indicated that menthol cigarette smokers displayed a significantly lower NMR than non-menthol cigarette users (0.32 vs. 0.37, p<0.001).[46] Moreover, different studies found that menthol inhibited CYP2A6 activity in vitro [38] and nicotine metabolism in vivo.[29] Specifically, menthol inhibits the microsomal oxidation of nicotine to cotinine and the P450 2A6-mediated 7-hydroxylation of coumarin.[37]

Specific Aim 2: Measure and compare the urinary biomarkers of tobacco exposure (PAHs, VOCs, and metals) between exclusive menthol smokers and non-menthol smokers.

Hypothesis: We hypothesize that levels of urinary biomarkers of tobacco exposure differ by menthol status.

Justification: Menthol is a flavor additive with a minty taste that reduces the irritation and harshness of smoking.[17] Menthol's cooling sensation in cigarettes may allow smokers to inhale more deeply and result in higher biomarkers of toxicant exposure.[100]

Specific Aim 3: Explore the effects of menthol on gradually reduced VLNC cigarettes on blood cotinine and smoking behavior (cigarettes smoked per day).

Hypothesis: We hypothesize that the treatment effects of gradually reduced VLNC cigarettes on blood cotinine and smoking behavior (cigarettes smoked per day) would be smaller in menthol smokers than non-menthol smokers.

Justification: Among both menthol and non-menthol smokers, participants assigned to the VLNC treatment groups exhibited less cigarettes smoking and toxicant exposure than participants randomized to the UNC group.[86] However, Hatsukami et al. found a significant nicotine content by menthol status interaction. Specifically, the effect of cigarette nicotine content on the number of cigarettes smoked per day was smaller for menthol smokers than non-menthol smokers.[86,93]

Chapter 3

Effect of Menthol on Nicotine Metabolite Ratio and Biomarkers of Tobacco Exposure

3.1 Introduction

Although the prevalence of cigarette smoking has decreased from 20.9% (2005) to 14.0% (2019),[1] cigarette smoking still remains the primary preventable cause of death in the United States (U.S.) and is responsible for more than 480,000 deaths annually.[2] Menthol, a monocyclic terpene alcohol, is widely used by tobacco companies as a characterizing flavor in cigarettes.[14,101] Menthol plays an important role in combining certain neuronal receptors that regulate pain, taste and other sensation of brain, which contributes to its unique proprieties in cooling, and analgesic.[14] Many tobacco experts have criticized the use of menthol as an additive in cigarettes because it hides the unpleasant harshness or bitter taste of cigarette smoke and aversive physiological effects of smoking.[15,16] Due to its "masking" function, menthol in cigarettes may facilitate smokers' exposure to nicotine, [17-19] and the likelihood of smoking initiation especially among Black youths.[15,20-24,102]

Multiple studies suggest that menthol may inhibit the metabolism of nicotine,[38] which further impacts the total metabolic clearance of nicotine.[29] Nicotine metabolite ratio (NMR), calculated as the ratio of 3′ hydroxycotinine (3HC) divided by cotinine,[103] is a measure of nicotine metabolic activity and a stable indicator of CYP2A6,[42] a liver enzyme that mediates the metabolization from nicotine to cotinine and cotinine to 3HC.[40,41] The research conducted by Fagan et al. found that young adult daily menthol smokers presented significantly lower NMR than non-menthol smokers.[30] However, there is an ongoing debate regarding the menthol effect

on NMR. For instance, Ho et al. found that menthol cigarettes did not significantly impact the NMR after adjusting for age, gender, and body mass index (BMI).[47] Another case-control study also revealed that there was no difference in NMR between menthol and non-menthol smokers after controlling for race.[48]

Mainstream cigarette smoke contain more than 7,000 chemical compounds in cigarette smoke.[2] Some of them are well-known human carcinogens and others are toxic agents that contribute to smoking-related illness, such as heart disease and lung disease.[2] A few studies have investigated the differences between menthol and non-menthol cigarette smokers in biomarkers of tobacco smoke such as carbon monoxide (CO) and tobacco-specific nitrosamines. [48,53-59] Some of them indicated menthol cigarette users had higher levels of cotinine and CO than non-menthol smokers, [48,53,57-59] others concluded that smokers of both menthol and non-menthol cigarettes presented similar levels of nicotine and nitrosamine biomarkers of smoke exposure. [56,104] Jones et al. found that menthol cigarette users presented significantly higher levels of blood cadmium (Ratio of geometric mean; Menthol/Non-menthol: 1.10 [95% CI: 1.04-1.16]) compared to non-menthol smokers. [60] Both PAHs and VOCs are included in the U.S. FDA's harmful and potentially harmful constituents (HPHCs) list. [105] PAHs are usually generated by incomplete combustion of tobacco and other organic components during smoking, [81] and benzo[a]pyrene (BaP) is classified as a Group 1 carcinogen, one of the most potent carcinogens. [82,106] Tobacco smoke is the main source of non-occupational exposure to harmful VOCs in the United States. [107] Toxic and carcinogenic VOCs such as acrylonitrile and benzene [108] have contributed to different tobacco-related cancers and non-cancer disease risk. [109-111]

The National Health and Nutrition Examination Survey (NHANES) aims to assess the health and nutrition status of adults and children in the United States. The NHANES is a publicly available dataset with a stratified, multi-stage, random probability sampling design conducted by the National Center for Health Statistics (NCHS) at the Center for Disease Control and

Prevention (CDC) in the United States.[71] NHANES provides a nationally representative sample of the civilian, noninstitutionalized U.S. population. The NHANES study protocol was approved by the National Center for Health Statistics Institutional Review Board, and all participants provided oral and written informed consent. [71] The NHANES started to provide 3'-hydroxycotinine and cotinine measurements in 2013 and the data on special smoker sample with urinary biomarker concentrations became available for the first time with 2015-2016 wave of the survey.

The first objective of this study was to compute urine NMR in menthol and non-menthol cigarette smokers using NHANES 2015-2016 special sample wave. The second objective was to examine urine metabolites of PAHs, VOCs, and metals between menthol and non-menthol cigarette smokers.

3.2 Materials and Methods

Study population

NHANES 2015-2016 obtained data from both smokers and nonsmokers. Participants aged 18 years and older from one-third of NHANES sample were chosen for urine collection. NHANES 2015-2016 special sample consists of those participants aged 18 years or older within the one-third subsample of NHANES 2015-2016 and all adult smokers aged 18 years or older. Adult smoker was defined as participant who smoked at least 100 cigarettes lifetime and now smoke cigarettes every day.[71] To be eligible for analysis, participants must have answered cigarette use and recent tobacco use survey, have been classified as either a menthol cigarette or non-menthol cigarette smoker, and must have reported non-missing cigarette menthol indicator information. We excluded smokers who had used any other tobacco products (pipes, hookah,

cigars, e-cigarettes, smokeless tobacco, or snuff) within the last 5 days prior to the visit. After excluding observations with missing data for other covariates used in this study, the final analytic sample consists of 351 exclusive cigarette smokers, of which 46.0% (162/351) smoked menthol cigarettes and 54.0% (189/351) smoked non-menthol cigarettes.

Participant smoking characteristics and sociodemographic measures

Information on smoking status, cigarettes smoked per day, cigarette rod length, cigarette menthol indicator, and Federal Trade Commission (FTC) nicotine content was obtained from cigarette use and recent tobacco use surveys. Cigarette rod length was categorized as regular/king (68-72mm,79-88mm) and long/ultra-long (94-101mm,110-121mm).[112] Sociodemographic variables such as age at screening (continuous), gender (categorical, male vs. female), race/ethnicity (categorical, Non-Hispanic White vs. Non-Hispanic African American vs. Hispanic and all others), education attainment (categorical, less than high school vs. high school or higher), weight (continuous, kg), height (continuous, cm), body mass index (BMI, kg/m^2), and ratio of family income to poverty (continuous) were collected through surveys administered in the mobile examination centers (MEC).

Analysis of tobacco related biomarkers and laboratory methodology

Urine nicotine, urine cotinine, and urine 3HC tobacco related biomarkers were available in the NHANES 2015-2016 special sample. Cotinine and 3HC are the two predominant nicotine metabolites in urine. Due to their greater concentrations and longer elimination half-lives, [40] cotinine and 3HC are preferred biomarkers of tobacco exposure. NMR is defined as the ratio of 3HC and cotinine.[103] NMR has been used as a well-established non-invasive probe for the index

of cytochrome P-450 2A6 activity (CYP2A6).[103] Urine metabolites of PAHs, VOCs and metal biomarkers were also available in the NHANES 2015-2016 special sample. Although NHANES provided VOCs data in blood, urine biomarkers of VOCs presented longer half-life and more stable property in storage and handling.[113] Appendix Table A-2 listed all 15 urine metabolites of VOCs analyzed in our study including their parent compound and common names. For metals, we included cadmium, cobalt, lead, and uranium biomarkers. All urine metabolites of PAHs, VOCs and metal biomarker concentrations were corrected for dilution by creatinine and were reported per gram of creatinine.[112]

For biomarker with analytic results below the lower limit of detection, an imputed fill value was placed in the analyte results filed by NHANES. This value is the lower limit of detection (LLOD) divided by the square root of 2 (LLOD/sqrt[2]). [112]

Statistical Analysis

The relationship between the categorical and continuous descriptive variables were analyzed using the Rao-Scott $\chi 2$ test and two-sample t-test. Multivariable linear regression models were used to obtain the covariates adjusted geometric means for NMR and tobacco related biomarkers. We estimated multivariable adjusted ratios of geometric means of biomarkers of tobacco exposure (urine nicotine, urine cotinine, urine 3HC) and NMR comparing smokers of menthol cigarettes with non-menthol cigarette smokers. The ratios of the geometric means and their 95% CIs were obtained by exponentiation from the linear regression models on log-transformed biomarker levels.[60] Next, we further evaluated the multivariable-adjusted ratios of geometric means of biomarkers of tobacco exposure and NMR comparing smokers of menthol cigarettes to non-menthol cigarette smokers stratified by race/ethnicity. All biomarkers were natural log-transformed to better fit the regression assumptions and analysis models.[112] We

estimated multivariable adjusted ratios of geometric means of biomarkers of tobacco exposure (PAHs, VOCs, and metals) comparing smokers of menthol cigarettes with non-menthol cigarette smokers. In addition to log transformation, PAHs, VOCs, and metals were creatinine-corrected to obtain covariates adjusted geometric means from the regression models. We included following covariates to control for any potential confounding effects: gender, race/ethnicity, education, cigarette rod length,[112] age at screening, BMI, ratio of family income poverty, FTC nicotine, and cigarettes per day (CPD).

SAS statistical software version 9.4 (SAS Institute) was used to conduct all statistical analyses using two-sided significance level of 0.05. SAS SURVEY Procedures (PROC SURVEYMEANS, PROC SURVEYFREQ, PROC SURVEYREG) were used to perform all statistical analyses with appropriate weights (from the NHANES 2015-2016 special sample), strata, and clustering variables to account for the complex sampling design of NHANES and to obtain nationally representative estimates.[71,72] Unweighted frequencies and weighted percentages were listed in all tables for ease of interpretation of the study findings.

3.3 Results

Table **3-1** summarizes the sample characteristics of the menthol and non-menthol cigarette smokers. Of 351 exclusive cigarette smokers, 46.0% (162/351) smoked menthol cigarettes and 54.0% (189/351) smoked non-menthol cigarettes. The gender distribution was similar among exclusive cigarette smokers. There were significant differences between menthol and non-menthol cigarette smokers by race/ethnicity, education attainment, age at screening, FTC nicotine, and cigarettes per day. Menthol smokers were more likely to be younger (42 vs. 48), have less than a high school education (24% vs. 14%), smoke less cigarettes per day (11 vs. 14) than non-menthol cigarette users.

Table **3-1.** Menthol versus non-menthol cigarette smokers' demographics

	Menthol cigarette smoker (n=162)	Non-menthol cigarette smoker (n=189)	p-value*
Gender			0.43
Male	94(52.0[41.3,62.8])	108(47.2 [39.1,55.3])	
Female	68(48.0[37.2,58.7])	81(52.8[44.7,60.9])	
Race/ethnicity			**<0.0001**
Mexican American/Hispanic	25(12.3 [6.3,18.4])	53(14.2[6.2,22.1])	
Non-Hispanic White	36(45.1[34.0,56.2])	85(70.9[58.2,83.7])	
Non-Hispanic Black	91(38.2[26.5,49.9])	27(6.4[0.1,12.7])	
Other Race-Including multi-racial	10(4.4[1.9,7.0])	24(8.5[4.1,13.0])	
Education			**0.03**
Less than HS	47(23.6[14.4,32.7])	41(14.4[9.0,19.8])	
HS or higher	115(76.4[67.3,85.6])	148(85.6[80.2,91.0])	
Cigarette rod length			0.31
Regular/king (68-72mm,79-88mm)	84(54.9[37.7,72.1])	121(63.6[54.3,72.9])	
Long/ultra-long(94-101mm,110-121mm)	78(45.1[27.9,62.3])	68(36.4[27.1,45.7])	
Age (in years) at screening	42.2(40.4,44.0)	47.7(45.3,50.0)	**0.0009**
Standing height (cm)	169.7(167.9,171.4)	168.3(166.4,170.3)	0.23
Weight (kg)	85.8(80.0,91.5)	81.6(77.7,85.5)	0.06
BMI (kg/m^2)	29.7(27.9,31.6)	28.7(27.5,29.9)	0.21
Ratio of family income to poverty	2.2(1.7,2.7)	2.5 (2.1,2.8)	0.32
FTC nicotine (mg)	1.1(1.0,1.1)	0.9(0.8,1.0)	**0.01**
Urine NMR	2.5(2.0,3.0)	2.0(1.7,2.2)	0.09
CPD	10.6(9.2,12.0)	13.8(12.9,14.7)	**0.0004**

Table **3-2** shows sociodemographic characteristics of cigarette smokers by race/ethnicity. There were significant differences in gender, education attainment, cigarette flavor, age at screening, FTC nicotine, serum cotinine, and cigarettes per day among Non-Hispanic White (NHW), Non-Hispanic Black (NHB), and Hispanics and all others (HISPO). NHW smokers were more likely to be female, have a high school or higher education, have a higher ratio of family income to poverty, and to smoke more cigarettes per day compared to NHB and HISPO. More than 75% of NHB smoked menthol cigarettes, and they were more likely than NHW smokers to smoke fewer cigarettes per day (8 for NHB vs. 15 for NHW) yet present substantially higher serum cotinine levels (270 ng/ml for NHB vs. 244 ng/ml for NHB) as reported in Table **3-2**.

Table **3-2.** Sociodemographic characteristics of cigarette smokers by race/ethnicity

	Non-Hispanic White (n=121)	Non-Hispanic Black (n=118)	Hispanics and all others (n=112)	p-value
Gender				**0.0089**
Male	58(42.1[32.4,51.7])	72(52.7[39.3,66.2])	72(66.6[54.7,78.6])	
Female	63(57.9[48.3,67.6])	46(47.3[33.8,60.7])	40(33.4[21.4,45.3])	
Education				**0.0004**
Less than HS	17(10.0[3.8,16.3])	35(30.0[21.7,38.3])	36(30.5[16.6,44.4])	
HS or higher	104(90.0[83.7,96.2])	83(70.0[61.7,78.3])	76(69.5[55.6,83.4])	
Cigarette flavor				**<0.0001**
Menthol	36(28.4[18.4,38.4])	91(78.9[66.7,91.0])	35(31.5[18.4,44.5])	
Non-menthol	85(71.6[61.6,81.6])	27(21.1[9.0,33.3])	77(68.5[55.5,81.6])	
Cigarette rod length				0.22
Regular/king (68-72mm,79-88mm)	74(63.7[52.5,75.0])	63(51.3[36.0,66.6])	68(58.0[44.7,71.4])	
Long/ultra-long(94-101mm,110-121mm)	47(36.3[25.0,47.5])	55(48.7[33.4,64.0])	44(42.0[28.6,55.3])	
Age (in years) at screening	46.8(44.3,49.2)	45.4(42.8,47.9)	42.1(38.8,45.4)	**0.0411**
Standing height (cm)	168.4(165.8,171.0)	170.3(168.8,171.9)	168.8(166.7, 170.9)	0.33
Weight (kg)	81.1(76.1,86.1)	86.8(81.3,92.2)	86.1(80.7,91.6)	0.06
BMI (kg/m^2)	28.5(27.1,29.9)	29.8(27.9,31.8)	30.1(28.5,31.7)	0.12
Ratio of family income to poverty	2.8 (2.4,3.2)	1.7(1.2,2.2)	1.6(1.4,1.9)	**<0.0001**
FTC nicotine (mg)	1.0(0.9,1.0)	1.1(1.0,1.1)	1.0(0.9,1.0)	**0.0005**
Serum cotinine (ng/ml)	244.1(213.1,275.2)	269.6(253.2,286.0)	160.5(136.1,184.9)	**<0.0001**
CPD	14.6(13.4,15.8)	8.4(7.6,9.1)	10.3(8.6,12.1)	**<0.0001**

Table **3-3** shows the adjusted geometric means for tobacco smoke exposure biomarkers and NMR. After adjusting for gender, race/ethnicity, education, cigarette rod length, age, BMI, FTC nicotine and cigarettes per day, there were no differences in urine nicotine [Ratios of GMs (95% CI): 0.9 (0.7,1.3), p> 0.1], urine cotinine [Ratios of GMs (95% CI): 1.0 (0.8,1.3), p> 0.1], and urine 3HC (Ratios of GMs (95% CI): 1.1 (0.9,1.6), p> 0.1) between menthol and non-menthol cigarette smokers. Adjusted geometric means for NMR were 1.8 and 1.6 for menthol and non-menthol cigarette smokers, respectively. There was no difference in NMR by cigarette flavor (Ratio of GMs: 1.1; 95% CI: 0.8,1.6; p>0.1). We further conducted analysis for each racial/ethnic group to test the differences in biomarkers of tobacco smoke exposure between menthol and non-menthol smokers (Table **3-4**). Among each race/ethnic group, there were no differences in nicotine, cotinine, and 3HC between menthol and non-menthol cigarette smokers. NMR was higher in menthol cigarette smokers than non-menthol users among NHW (1.7 vs. 1.4) and HISPO (1.6 vs. 1.5), but the difference was not statistically significant (p>0.1; Table **3-4**). Among NHB smokers, NMR was lower in menthol users than non-menthol users (2.4 vs. 2.6) but still not approached significance in the covaries adjusted model (p>0.1; Table **3-4**).

Table **3-3.** Adjusted geometric means (GMs) for biomarkers and nicotine metabolite ratio in urine by menthol and non-menthol status

	Menthol Mean (95% CI)	Non-menthol Mean (95% CI)	Ratio of GMs (95% CI)	p
Biomarker*				
Nicotine (µg/g creatinine)	980(829,1160)	1050(844,1310)	0.9(0.7,1.3)	0.64
Cotinine (µg/g creatinine)	2250(1870,2700)	2240(1950,2560)	1.0(0.8,1.3)	0.96
3HC (µg/g creatinine)	3980(3410,4650)	3460(2730,4390)	1.1(0.9,1.6)	0.34
NMR	1.8(1.5,2.1)	1.6(1.3,1.9)	1.1(0.8,1.6)	0.37

* Adjusted for gender, race/ethnicity, education, cigarette rod length, age, BMI, FTC nicotine and cigarettes per day. Corrected for urinary creatinine.

Table **3-4.** Mean biomarkers for each race/ethnicity among menthol and non-menthol cigarette smokers

Biomarker*	Menthol Mean (95% CI)	Non-menthol Mean (95% CI)	Ratio of GMs (95% CI)	p
Non-Hispanic White				
Nicotine (µg/g creatinine)	1810(1380,2370)	1980(1470,2660)	0.9(0.6,1.3)	0.63
Cotinine (µg/g creatinine)	3660(2880,4650)	3680(2940,4620)	1.0(0.7,1.3)	0.97
3HC (µg/g creatinine)	6100(1830,7700)	5240(3920,7008)	1.2(0.9,1.6)	0.29
NMR	1.7(1.2,2.3)	1.4(1.1,1.9)	1.2(0.8,1.7)	0.37
Non-Hispanic Black				
Nicotine (µg/g creatinine)	955(773,1180)	710(480,1045)	1.3(0.8,2.2)	0.21
Cotinine (µg/g creatinine)	1720(1530,1920)	1890(1570,2300)	0.9(0.7,1.1)	0.38
3HC (µg/g creatinine)	4049(3610,4540)	4730(3980,5620)	0.9(0.7,1.1)	0.20
NMR	2.4(2.1,2.8)	2.6(1.9,3.4)	0.9(0.7,1.3)	0.75
Hispanics and all others				
Nicotine (µg/g creatinine)	548(315,952)	774(614,976)	0.7(0.4,1.4)	0.28
Cotinine (µg/g creatinine)	1980(1360,2903)	1880(1590,2220)	1.1(0.7,1.5)	0.74
3HC (µg/g creatinine)	3180(2087,4840)	2706(1909,3830)	1.2(0.8,1.7)	0.37
NMR	1.6(1.2,2.1)	1.5(1.2,1.8)	1.1(0.8,1.6)	0.59

* Adjusted for gender, education, cigarette rod length, age, BMI, and cigarettes per day. Corrected for urinary creatinine.

Table **3-5** lists the adjusted geometric means of urine metabolites of PAHs biomarkers by menthol status. There were no statistically significant differences between menthol and nonmenthol cigarette users in the 6 out of 7 creatinine-corrected metabolites of PAHs biomarkers. Menthol cigarette smokers had significantly lower 1-hydroxyphenanthrene of creatinine-corrected PAHs concentrations compared to non-menthol cigarette smokers (167.2 µg/g creatinine vs. 198.1µg/g creatinine; Ratio of GMs [95% CI]: 0.8 [0.7,1.0]; p-value: 0.03).

Table **3-6** lists the adjusted geometric means of urine metabolites of VOCs by menthol status. There were no statistically significant differences in most of (12/15) urine metabolites of VOCs between menthol nonmenthol cigarette smokers. Menthol cigarette smokers presented significantly lower concentrations of acrylamide (115.6 µg/g creatinine vs.154.6 µg/g creatinine; Ratio of GMs [95% CI]: 0.7 [0.6,0.9]; p-value: 0.02), N, N-dimethylformamide (403.1 µg/g creatinine vs.452.5 µg/g creatinine; Ratio of GMs [95% CI]: 0.9 [0.8,0.9]; p-value: 0.01), and acrylonitrile (120.9 µg/g creatinine vs.143.6 µg/g creatinine; Ratio of GMs [95% CI]: 0.8 [0.7,0.9]; p-value: 0.04) than nonmenthol cigarette smokers (Table **3-6**).

There were no statistically significant differences in all urine metals between menthol and non-menthol cigarette smokers after adjustment of covariates in cadmium (Ratio of GMs [95% CI]: 0.9[0.7,1.1]; p-value:0.21), cobalt(Ratio of GMs [95% CI]: 1.1[0.9,1.3]; p-value:0.4), lead(Ratio of GMs [95% CI]: 0.9[0.8,1.1]; p-value:0.18), and uranium(Ratio of GMs [95% CI]: 1.2[0.9,1.6]; p-value:0.11).

Table **3-5.** Adjusted Geometric Means of Polycyclic Aromatic Hydrocarbons by menthol status

	Units	Menthol Mean (95% CI)	Nonmenthol Mean (95% CI)	Ratio of GMs (95% CI)	p
Polycyclic Aromatic Hydrocarbons*					
1-Hydroxynapthalene	µg/g creatinine	10.2(8.8,11.8)	11.5(9.7,13.7)	0.9(0.7,1.1)	0.16
2-Hydroxynapthalene	µg/g creatinine	14.3(13.0,15.7)	14.7(13.1,16.4)	1.0(0.8,1.1)	0.69
3-Hydroxyfluorene	ng/g creatinine	638.7(561.7,726.4)	699.7(593.7,824.8)	0.9(0.7,1.2)	0.37
2-Hydroxyfluorene	ng/g creatinine	1002.0(903.3,1111.8)	1114.1(990.5,1253.1)	0.9(0.8,1.1)	0.19
1-Hydroxyphenanthrene	ng/g creatinine	167.2(148.7,188.1)	198.1(172.6,227.4)	0.8(0.7,1.0)	**0.03**
1-Hydroxypyrene	ng/g creatinine	269.5(238.7,304.2)	294.3(246.4,351.6)	0.9(0.8,1.1)	0.32
2-Hydroxyphenanthrene & 3-Hydroxyphenanthrene	ng/g creatinine	292.5(253.7,337.1)	337.0(283.3,400.9)	0.9(0.7,1.0)	0.09

* Adjusted for gender, race/ethnicity, education, cigarette rod length, age, BMI, Ratio of family income to poverty, FTC nicotine, NMR, and cigarettes per day.

Table **3-6.** Adjusted Geometric Means of Volatile Organic Compound metabolites by menthol status

Volatile organic compound metabolites*		Units	Menthol Mean (95% CI)	Nonmenthol Mean (95% CI)	Ratio of GMs (95% CI)	p
Parent Compound	Common Name					
Xylene	2-MHA	µg/g creatinine	103.9(86.8,124.5)	109.2(97.0,122.9)	1.0(0.8,1.2)	0.64
Xylene	3-MHA and 4-MHA	µg/g creatinine	635.3(541.4,745.6)	692.0(616.3,777.0)	0.9(0.7,1.1)	0.40
Acrylamide	AAMA	µg/g creatinine	115.6(103.3,129.4)	154.6(123.7,193.2)	0.7(0.6,0.9)	**0.02**
N, N-Dimethylformamide	AMCC	µg/g creatinine	403.1(370.3,438.9)	452.5(412.9,495.7)	0.9(0.8,0.9)	**0.01**
Cyanide	ATCA	µg/g creatinine	154.1(130.8,181.6)	152.9(131.5,177.7)	1.0(0.8,1.3)	0.94
Toluene	BMA	µg/g creatinine	7.7(5.8,10.1)	7.5(6.5,8.6)	1.0(0.7,1.4)	0.87
1-Bromopropane	BPMA	µg/g creatinine	4.4(3.1,6.2)	4.7(3.9,5.7)	0.9(0.6,1.4)	0.69
Acrolein	CEMA	µg/g creatinine	256.6(227.6,287.1)	259.9(227.1,297.5)	1.0(0.8,1.2)	0.86
Acrolein	3HPMA	µg/g creatinine	997.8(863.2,1153.4)	1103.3(951.9,1279.0)	0.9(0.7,1.1)	0.30
Acrylonitrile	CYMA	µg/g creatinine	120.9(103.7,141.0)	143.6(124.4,165.9)	0.8(0.7,0.9)	**0.04**
1,3-Butadiene	DHBMA	µg/g creatinine	442.6(405.2,483.5)	438.7(403.7,476.8)	1.0(0.9,1.2)	0.89
Propylene oxide	2HPMA	µg/g creatinine	70.0(60.2,81.4)	71.1(62.4,81.0)	1.0(0.8,1.2)	0.87
Styrene	MA	µg/g creatinine	267.8(236.2,303.8)	275.9(238.4,319.3)	1.0(0.8,1.1)	0.70
Ethylbenzene, styrene	PGA	µg/g creatinine	325.0(279.2,378.2)	331.3(280.1,391.8)	1.0(0.8,1.2)	0.84
Crotonaldehyde	HPMMA	µg/g creatinine	1071.5(934.8,1228.2)	1247.6(1110.3,1401.8)	0.9(0.7,1.0)	0.11

* Adjusted for gender, race/ethnicity, education, cigarette rod length, age, BMI, Ratio of family income to poverty, FTC nicotine, NMR, and cigarettes per day.

3.4 Discussion

NMR has been considered as a reliable predictor of smoking cessation outcomes in a few clinical studies.[45] Instead of using the same and general treatment for all smokers, personalized cessation pharmacotherapies based on smokers' NMR has been found successful with higher efficiency[50,51] and lower cost.[50] Most studies investigated serum NMR with serum cotinine and 3HC, but few of them computed NMR in urine with urinary biomarkers. The NHANES started to provide 3'-hydroxycotinine and cotinine measurements in 2013 and the data on special smoker sample with urinary biomarker concentrations became available for the first time with 2015-2016 wave of the survey. NMR tailored cessation treatments usually lead to maximum treatment response and minimum side effects of cessation aid.[50] Therefore, it is imperative to delineate the potential factors that may affect urine NMR. Multiple studies suggest that menthol may negatively impact metabolism of nicotine through inhibition of CYP2A6 activity[38] and clearance of nicotine.[29] Nonetheless, it still remains controversial regarding the roles played by menthol in nicotine metabolism. Using the nationally representative sample of smokers, our results show urine NMR did not differ between menthol and non-menthol cigarette smokers.

Both PAHs and VOCs are listed as FDA's HPHCs in tobacco. [105] Due to the inherent toxicity and carcinogenicity of PAHs and VOCs, it is important to consider the differences in exposure to these substances among mentholated tobacco users. We quantified urine metabolites of 7 PAHs, 15 VOCs, and 4 metals collected from NHANES 2015-2016 special sample wave by cigarette smokers' flavor status. We found that one metabolite of PAHs (1-hydroxyphenanthrene) and three metabolites of VOCs (acrylamide, N, N-dimethylformamide, and acrylonitrile) biomarkers were significantly lower among menthol cigarette smokers compared to non-menthol cigarette smokers. No differences by flavor were found for the 22 other markers. PAHs are a class

of compounds composed of two or more fused benzenoid rings, which lead to mutagenic effect. [114] As indicated by the International Agency for Research on Cancer (IARC), several PAHs in tobacco smoke are known for human carcinogens, [106] phenanthrene and its metabolite 1-hydroxyphenanthrene are strongly correlated with other PAHs in smoke and urine, respectively. [112,114] Further, acrylonitrile, a Group 2B carcinogen as classified by IARC, is possibly carcinogenic to human. [115] However, there are no differences between menthol and non-menthol cigarette smokers in most of urine metabolites of PAHs and VOCs biomarkers as shown in result section. For instance, 1,3-Butadiene is responsible for hematological malignancies. [116] Acrolein, one of the major tobacco chemicals, is a potent cardiopulmonary toxicant which contributes to increased risk of cardiovascular disease [117] and potentially induces gene mutations of lung cancer. [118] Menthol and non-menthol cigarette smokers had similar levels of 1,3-Butadiene and acrolein metabolites of VOCs biomarkers. In addition to PAHs and VOCs, it was previously reported in studies of NHANES that exposure to the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), another IARC classified lung carcinogen, [77,78,119] does not appear to differ by menthol status as determined by its urinary biomarker 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol NNAL. [55,56]

It is also worth noting that although menthol cigarette users presented relatively lower concentrations of certain metabolites of PAHs and VOCs in the present study, the differences in most biomarkers of tobacco exposure toxicants are still not statistically significant. Further, we need to consider the following limitations. Cigarette brand is classified as menthol and non-menthol by its manufacturer, but most cigarettes contain low levels of menthol even if they are nonmenthol brands. [104,120] Also, there may be other cigarette design characteristics besides menthol flavor that can affect the exposure to tobacco smoke constituents. [121] The major public health burden of menthol flavor is its role in facilitating smoking initiation among youth. [15,20-24] Menthol may facilitate increased tobacco smoke inhalation due to its cooling properties, but we

did not find direct evidence of this effect in the current study as shown by similar cotinine levels with non-menthol smokers.

Chapter 4

Effect of Menthol on Nicotine Reduction: Pooled results from two Double-Blind Randomized Controlled Trials

4.1 Introduction

On April 29, 2021, United States (U.S.) Food and Drug Administration (FDA) announced its commitment to ban menthol as a characterizing flavor in cigarettes and ban all characterizing flavors (including menthol) in cigars within the next year. Menthol in cigarettes may facilitate smoking due to its cooling sensation and reduced harshness. [120,122] A simulation model based on the data from the National Health Interview Survey (NHIS) estimated that menthol cigarettes contributed to more than 10 million extra smokers, 3 million life years lost and at least 350,000 premature deaths from 1980 to 2018. [123] Black smokers are more likely to use menthol cigarettes, which reflects decades of targeted marketing of menthol cigarettes to blacks. [124-126]

In addition to menthol flavor, nicotine reduction is another possible strategy considered by FDA to decrease prevalence of cigarette smoking. [99] In 2018, the U.S. FDA issued an Advanced Notice of Proposed Rulemaking (ANPRM) to reduce nicotine in tobacco products to make them minimally addictive or nonaddictive. [85] Trials of reduced nicotine content (RNC) cigarettes have found multiple benefits, including reduced nicotine intake and lower toxicant exposure levels and minimal harmful health effects. [86,88-92,127,128] There are two strategies (immediate and gradual nicotine reduction) considered by FDA to reduce nicotine content to minimal levels in cigarettes. [85] In clinical trials, both immediate and gradual nicotine reduction resulted in significant decreases in biomarkers of smoke exposure. [86,90]

In the current study we evaluated whether menthol modifies the treatment effect of a gradual reduction from fully nicotinized cigarettes to very low nicotine cigarette (VLNC 0.4mg/cig) over a 5-month period of time. A critical consideration in trials designed to guide policy is cotinine reduction, cigarettes per day (CPD), nicotine dependence, expired carbon monoxide (CO), and study compliance to smoking VLNC in the treatment groups. While lowering nicotine and smoke exposure using RNC is desirable, how smokers respond to smoking VLNC, or non-addicting cigarettes is critical for smoking cessation. In theory, the lack of reinforcing effects from cigarettes with minimal nicotine should facilitate cessation. In trials evaluating switching directly or through RNC stepped down methods to VLNC, smokers were encouraged but not required to smoke low nicotine cigarette (including VLNC) exclusively. If they were unable to comply and also smoked their regular cigarettes, they were asked to report non-compliance. Compliance to using VLNC study cigarettes is a major objective of RNC trials since only VLNC are considered minimally or not addicting. If smokers are able to smoke VLNC without significant withdrawal symptoms and resorting to smoking their regular cigarettes, their ability to quit smoking altogether is expected to be increased substantially.

We hypothesized that the treatment effects of gradually reducing nicotine from commercial to VLNC research cigarettes on reducing smoking exposure including cigarettes per day and nicotine (e.g., as measured by cotinine) would be smaller in menthol smokers than non-menthol smokers.

4.2 Materials and methods

Study Design and Study Population

We included two randomized clinical trials of progressive nicotine reduction for this analysis. The first one was a two-site, two-arm, double-blind, parallel-group, randomized clinical trial conducted at the Penn State University (PSU) College of Medicine (Hershey, PA, USA) and George Washington University (GWU) between 2015 and 2018. [94] The second one was also a two-site, two-arm, double-blind, parallel-group, randomized clinical trial conducted at the Penn State College of Medicine (Hershey, PA, USA) and Massachusetts General Hospital (MGH). [95] Both trials were managed under pharmacy-controlled protocols and were conducted to determine the effects of nicotine reduction in populations with high rates of smoking. The PSU-GWU trial included cigarette smokers with low socioeconomic status (SES; less than a college degree), and the PSU-MGH trial included smokers with the mental health (MH) conditions (mood and/or anxiety disorders) as determined by the structured Mini-International Neuropsychiatric Interview. [94,95] Table **4-1** listed inclusion and exclusion criteria of PSU-GWU trial.[94] Table **4-2** listed inclusion and exclusion criteria of PSU-MGH trial.[95] Both trials consisted of 4 phases (baseline 1, baseline 2, randomized, and treatment choice) with multiple clinic visits (for a total of 11 and 12 visits for SES and MH, respectively) at the study centers over 33 weeks. [94,95] Baseline1 included smoking participants' usual brand cigarettes for one week. Baseline 2 included two weeks of smoking usual nicotine content (UNC) study cigarettes (nicotine content approximately 11.6 mg/cigarette). In the 18-week randomized phase, participants were randomized to the control arm in which they continued on the UNC study cigarettes, or the intervention arm to receive progressively reduced nicotine content (RNC) study cigarettes. For both trials, participants in the control group received SPECTRUM cigarettes with 11.6 mg nicotine/cigarette for the entire 18-

week period. Participants in the gradual group received SPECTRUM cigarettes with nicotine contents of 7.4, 3.3, 1.4, 0.7, and 0.2 (VLNC) mg nicotine/cigarette (3 weeks per dose from 7.4 to 0.7 mg nicotine/cigarette, 6 weeks for 0.2 mg nicotine/cigarette). [94,95] Participants in both trials were matched to menthol or non-menthol study cigarettes based on the flavor of their usual cigarettes. The randomized phase was followed by a 12-week treatment choice phase.

Table **4-1.** Inclusion and exclusion criteria (SES)[94]

Inclusion Criteria	Description
Age	18-65
Education	<16 years or < bachelor's degree
Cigarette frequency	≥ 5 cigarettes per day
Cigarette history	At least 12 months
Cigarette flavor	Willing to smoke menthol or non-menthol cigarette flavor
Smoking cessation	No intention to quit in the next 6 months
Accessibility	Accessibility to study centers and to receive phone calls for next 8 months
Exclusion Criteria	Description
1	Current pregnancy or nursing
2	Serious medical conditions
3	Prisoners or subject to correctional supervision
4	Systolic blood pressure ≥ 160 mmHg
5	Use of non-cigarette nicotine delivery product
6	Difficulty providing blood samples
7	Alcohol abuse
8	Participating in a trial related to reduced nicotine content cigarettes
9	Plan to take surgery in the next 8 months

Table **4-2.** Inclusion and exclusion criteria (MH)[95]

Inclusion Criteria	Description
Age	18-65
Residence	Live in the local area for the next 8 months
Cigarette frequency	≥ 4 cigarettes per day for at least 12 months
Quit attempt	No quit attempt or planning to quit in the next 6 months
Cessation aid	No use of varenicline, bupropion, nicotine patch, gum, lozenge
Diagnostic criteria	Current or lifetime unipolar mood disorder or anxiety disorder
Language	Able to read and write in English, understand and consent to study protocol
Exclusion Criteria	Description
1	Current pregnancy or nursing
2	Serious medical conditions
3	Prisoners or subject to correctional supervision
4	Systolic blood pressure ≥ 160 mmHg; heart attack; stroke; COPD; kidney or liver disease
5	Use of non-cigarette nicotine delivery product
6	Difficulty providing blood samples
7	Alcohol abuse
8	Participating in a trial related to reduced nicotine content cigarettes
9	Plan to take surgery in the next 8 months
10	Increased risk of suicide based on clinical assessment

Participants attended in-person visits during the randomization phase, and completed subjective responses to the study cigarettes (assessed through modified Cigarette Evaluation Questionnaire [96] and cigarette-liking scale [97,12901,130]). Furthermore, research coordinators collected blood samples and expired Carbon Monoxide (CO) to evaluate biomarkers of nicotine, toxicant exposure, and study compliance for both trials. [94,95] The study flow diagrams are shown in Figure **1-2** (SES; Chapter 1) and Figure **1-3** (MH; Chapter 1). Additional information about these two trials, such as study protocols, timeline, methods, randomization procedures, primary and secondary outcomes were published elsewhere. [90,94,95] All subjects provided written inform consent and the study was approved by the George Washington University (IRB #011507) and Penn State College of Medicine (STUDY #00000660) Institutional Review Boards. Both studies are registered at clinicaltrials.gov (SES: NCT01928719; MH: NCT01928758).

Measures

Smoking behaviors and nicotine biomarkers were evaluated through cigarettes per day (CPD), blood cotinine, and expired breath CO at the last visit of randomization phase for both trials (visit 9 for SES; visit 10 for MH). Participants were required to keep a daily cigarette log to record the number of study and non-study cigarettes smoked. We used an immunoassay kit and the piCO+ Smokerlyzer to collect and assess plasma cotinine (ng/ml) and expired CO (ppm). In addition, the Heaviness of Smoking Index (HSI) [130], the Fagerström Test for Cigarette Dependence (FTCD) [1-10] [131], and the Hooked-on Nicotine Checklist (HONC) [0-10] [132] were completed by participants during in-person visits.

To measure and assess the effect of menthol flavor on subjective responses to study cigarettes, we used the PhenX toolkit measure modified cigarette evaluation questionnaire (mCEQ, Likert scale: 1-7) [96] and cigarette-liking scale (CLS, Likert scale: 1-10 for CLS item 1-8;

1-5 for CLS item 9 and 10) [97,129]. The mCEQ is a 12-item assessment of the reinforcing, adverse effects, and enjoyment of smoking. The cigarette liking scale is a measure of the sensory properties of cigarettes including its taste and harshness.

Study compliance was defined according to Benowitz et al. method [V_{last} cotinine/CPD]/[$V_{baseline}$ cotinine/CPD] [133], we assessed the biochemical compliance in VLNC group only at the final randomization phase visit (visit 9 for SES, and visit 10 for MH). Additional details of method with adjustment for environmental tobacco smoke using a random sample from the current trials were previously reported [134]. Results on overall (not stratified by menthol) biochemical compliance in SES were reported previously[90]. Biochemical compliance is a measure appropriate for measuring VLNC where compensatory smoking does not affect nicotine intake at such low levels.

Table **4-3** and Table **4-4** listed descriptions of each variable used in the analysis of SES and MH trials.

Table **4-3.** Descriptions of each variable used in analysis (SES)

Outcome	**Name of Variable (SAS Analysis)**	**Type of Variable**	**Description**
Compliance	final_trial_compliance_ets_adj	Dichotomous	1. Composite of SRtrialcomply_indicator at V9 and final_totcig_biochem_comply_ets 2. Only applicable to RNG
CES subscale 1-5	ces.smoking.satisfaction; ces.psy.reward; ces.aversion; ces.enjoyment; ces.craving.reduction	Continuous	Cigarette Evaluation subscale: Smoking Satisfaction. Psychological Reward. Aversion. Enjoyment of Respiratory Tract Sensations. Craving Reduction
CLS 1-11	cls 1-11	Continuous	Cigarette Liking Scale
Blood cotinine	br_cot_elisa_p_lod	Continuous	Biomarkers: Cotinine – FINAL TRIAL VARIABLE Original Variable: br_cot_elisa_p Recoded Variable: br_cot_elisa_p_lod
Exhaled CO	exhaledCO_EW	Continuous	Bio-measures: Exhaled CO
Age	s1_age	Continuous	Age
Education	educ_cats.factor	Categorical	Less than a HS Degree; HS Degree; More than a HS Degree
Employment	currently.working	Categorical	Currently working: Yes; No
BMI	BMI.cat3.factor.v3	Categorical	Under/Normal Weight; Overweight; Obese;
Income	Income_cat.factor	Categorical	Low Income; Middle Income; High Income
Cigarettes Per Day	cpd.full.fb6.weekly.mean	Continuous	Cigarettes Per Day – Daily Cigarette Log
FTND	FTND_Score_JL	Continuous	the full definition of FTND – with CPD used in the calculation
PSUCDI	PSUCDI_Score_JL	Continuous	Penn State University Cigarette Dependence Index
HONC	total_HONC_Score_JL	Continuous	Hooked on Nicotine Checklist
Race	race.factor	Categorical	Race Categories Caucasian/white; African American/black; Other
Participant ID	part_id	N/A	Participant ID
Treatment	treatment	Categorical	U; R
Flavor	flavor_strata.factor	Categorical	Menthol; Non-menthol

Table **4-4.** Descriptions of each variable used in analysis (MH)

Outcome	Name of Variable (SAS Analysis)	Type of Variable	Description
Compliance	final_trial_compliance_ets_adj.factor	Dichotomous	Composite of SRtrialcomply_indicator at V10 and final_totcig_biochem_comply_ets
CES 1-12	ces1-12	Continuous	Cigarette Evaluation Scale
CLS 1-8	cls 1-8	Continuous	Cigarette Liking Scale
CLS 9- 10	cls 9-10	Continuous	Cigarette Liking Scale
Blood cotinine (Primary outcome)	br_cot_elisa_p_lod	Continuous	Biomarkers: Cotinine (Lisa Reinhart + Emily Wasserman) – FINAL TRIAL VARIABLE Original Variable: br_cot_elisa_p Recoded Variable: br_cot_elisa_p_lod
Exhaled CO (Secondary outcome)	exhaledCO_EW	Continuous	Bio-measures: Exhaled CO
QIDS Scoring (Depression)	qids_total_cat.factor	Categorical	Quick Inventory of Depressive Symptomatology None = 0-5; Mild = 6-10; Moderate = 11-15; Severe = 16-20; Very Severe = 21-27
OASIS Scoring (Anxiety)	oasis_total	Continuous	Overall Anxiety Severity and Impairment Scale
Age	s3_age	Continuous	Age at visit_1_arm_1
Education	educ_cats.factor	Categorical	High School Degree or Less; More than a High School Degree but Less than a Bachelor's Degree; Bachelor's Degree or More
Employment	employ_cats.factor	Categorical	Working Now Full-Time; Working Now Part-Time; Unemployed; Other;
BMI	bmi_cat3.factor	Categorical	Under/Normal Weight; Overweight; Obese;
Income	Income_cat.factor	Categorical	Low Income; Middle Income; High Income
Cigarettes Per Day	cpd.full.fb.weekly.mean	Continuous	Cigarettes Per Day – Daily Cigarette Log
FTND	FTND_Score_JL	Continuous	the full definition of FTND – with CPD used in the calculation
PSUCDI	PSUCDI_Score_JL	Continuous	Penn State University Cigarette Dependence Index
HONC	total_HONC_Score_JL	Continuous	Hooked on Nicotine Checklist
Race	demo37.factor	Categorical	Race Categories Caucasian/white; African American/black;

			Other
Unipolar mood and/or anxiety disorder	final_mini_diagnos_cats.factor	Categorical	Current Anxiety Disorder Only - Regardless of Past; Current Mood Disorder Only - Regardless of Past; Both Current Mood and Anxiety Disorders - Regardless of Past; No Current Mood or Anxiety Disorders - Past Mood Disorder Only; No Current Mood or Anxiety Disorders - Past Anxiety Disorder Only
Number of Years Smoked Cigarettes Daily	calc_yrs_smkd_daily	Continuous	Number of Years Smoked Cigarettes Daily
Participant ID	part_id	N/A	Participant ID
Treatment	treatment_rand.factor	Categorical	Usual; Reduced
Flavor	flavor_strata.factor	Categorical	Menthol; Non-menthol

Statistical Analyses

For the current analysis, we determined the effect of menthol on the final experimental condition (VLNC) at the last visit of randomization phase (after 6 weeks of smoking VLNC cigarettes at 0.2 mg nicotine/cigarette).

We used linear regression to investigate whether menthol flavor impacts the treatment effects of VLNC on changes to smoking measures including CPD, blood cotinine, expired breath CO, mCEQ, and CLS at the last visit of randomization phase for both trials. To measure and compare the treatment effects (defined as differences between VLNC and UNC groups) by menthol status, we included nicotine content treatment (VLNC or UNC) condition by cigarette flavor (menthol or non-menthol) interaction term in the linear regression models. [93] All linear regression models adjusted for baseline (visit 3 for SES, visit 4 for MH) measure of the outcomes, study, age (continuous, years), gender (categorical, male vs. female), race (categorical, white vs non-white), education (categorical, less than a HS degree or HS degree vs. more than a HS degree), employment status (categorical, currently working vs. not currently working), income (categorical; low ($0-$19,999), middle ($20,000-$59,999) and high (more than $60,000)) and other covariates (FTCD (continuous) or HSI[categorical; low, moderate, high addiction]). [90]

Study compliance was assessed through logistic regression model at the last visit of randomization phase for both trials in VLNC group only. Due to the limited sample size of VLNC groups in both trials (N= 62 for SES; N= 66 for MH), we combined these two trials to increase the sample size to 128 (62+66) since the definition and measurement process of compliance was consistent across both trials. The logistic regression model adjusted for study, age, gender, race, education, household income, and employment status.

Analyses were conducted by the Penn State TCORS Biostatistics and Database Management Core using statistical software SAS Version 9.4 (SAS Institute, Cary, NC, USA). All tests were two-sided at the 0.05 significance level.

4.3 Results

Baseline Characteristics by Study Cigarette Flavor Status

Of 245 cigarette smokers in SES trial, 45% were black and 53 % were women. About 70 % of participants were assigned to menthol-flavored study cigarettes based on their usual flavor preference (Table **4-5**). Menthol smokers had lower baseline exhaled CO (29 ppm vs. 38 ppm; p <0.001) compared to non-menthol smokers. Menthol smokers reported smoking fewer CPD than non-menthol smokers over the 2-week period (23 vs. 26; p=0.08; Table **4-5**).

Of 188 cigarette smokers in MH trial, about 40% of participants chose menthol-flavored study cigarettes (Table **4-5**). Menthol smokers had similar baseline exhaled CO (28 ppm vs. 30 ppm; p= 0.33) and CPD (20 vs. 21; p=0.41) compared to non-menthol smokers.

Table **4-5.** SES and MH trials baseline demographics by cigarette flavor

	SES			MH		
Characteristics	Menthol smokers (N=170)	Non-menthol smokers (N=75)	p-value	Menthol smokers (N=74)	Non-menthol smokers (N=114)	p-value
Age (years), mean (SD)	45.3(11.2)	43.7(11.9)	0.34	43.5(11.8)	43.2(12.8)	0.88
Gender (%)			0.74			0.73
Female	90(52.9)	38(50.7)		46(62.2)	68(59.7)	
Male	80(47.1)	37(49.3)		28(37.8)	46(40.4)	
Race (%)			**<0.0001**			**<0.0001**
White	83 (49.4)	70(93.3)		51(68.9)	95(83.3)	
Black	75(44.6)	3(4.0)		20(27.0)	6(5.3)	
Other	10(6.0)	2(2.7)		3(4.1)	13(11.4)	
Hispanic (%)	6(3.6)	0(0.0)	0.18	4(5.4)	4(3.5)	0.53
Education (%)			0.77			0.35
Less than a HS degree or HS degree	104(61.2)	48(63.2)		31(41.9)	40(35.1)	
More than a HS degree	66(38.8)	27(36.0)		43(58.1)	74(64.9)	
Household Income (%)			0.20			0.08
$0-19,999	51(41.5)	17(27.9)		28(45.9)	29(30.5)	
$20,000-59,999	45(36.6)	27(44.3)		19(31.2)	30(31.6)	
$60,000 +	27(21.9)	17(27.9)		14(22.9)	36(37.9)	
Treatment (%)			0.92			0.77
Reduced Nicotine	85(50.0)	37(49.3)		38(51.4)	56(49.1)	
Usual Nicotine	85(50.0)	38(50.7)		36(48.7)	58(50.9)	
Cigarettes/day, mean (SD)*	23.2(12.8)	26.2(11.7)	0.08	20.1(9.9)	21.4(11.2)	0.41
FTCD, mean (SD)	6.2(2.1)	6.5(2.1)	0.20	5.9(2.3)	6.1(2.3)	0.50
HONC, mean (SD)	6.9(2.4)	7.2(2.3)	0.32	8.1(2.1)	8.0(1.9)	0.83
Plasma Cotinine (ng/mL), mean (SD)	252.7(152.3)	278.8(138.5)	0.21	254.7(137.6)	248.4(143.9)	0.77
Exhaled carbon monoxide (ppm), mean (SD)	28.6(15.5)	37.9(16.9)	**<0.0001**	27.8(14.9)	30.2(17.4)	0.33

* The cigarette measures were the average for the 2-week Spectrum UNC study period.

Menthol and Pooled Analysis of Both Trials

Table **4-6** shows the pooled results from these two double-blind randomized controlled trials. Menthol flavor did not modify the treatment effects of VLNC on CPD, cotinine reduction, exhaled CO, and nicotine dependence ($p_i > 0.1$). No significant interactions were detected on mCEQ and CLS except CLS item 4 (Harsh, $p_i=0.01$). Among menthol smokers there is no difference in the rating of harsh score between VLNC and UNC. Among non-menthol smokers, VLNC rated the cigarettes being less harsh than UNC group.

Table **4-6.** Pooled results from two Double-Blind Randomized Controlled Trials

Outcome[1]	SES + MH				
	Menthol smokers		Non-menthol smokers		Interaction
	Treatment effect (95% CI) *	p-value	Treatment effect (95% CI) *	p-value	p-value
CPD	-6.6(-9.6, -3.6)	**<0.0001**	-3.9(-7.1, -0.8)	**0.02**	0.23
Cotinine	-157.1(-204.0, -110.3)	**<0.0001**	-207.5(-257.3, -157.7)	**<0.0001**	0.15
Exhaled CO	-6.9(-11.0. -2.7)	**0.001**	-5.1(-9.6, -0.7)	**0.02**	0.58
FTCD	-1.2(-1.7, -0.7)	**<0.0001**	-0.8(-1.3, -0.3)	**0.04**	0.26
HONC	-0.6(-1.2,0.04)	0.07	-0.6(-1.2,0.1)	0.08	0.95
mCEQ: Satisfaction	-0.8(-1.3, -0.3)	**0.002**	-0.8(-1.4, -0.3)	**0.004**	0.95
mCEQ: Reward	-0.5(-0.9, -0.1)	**0.007**	-0.6(-1.1, -0.2)	**0.003**	0.73
mCEQ: Aversion	-0.1(-0.3, 0.1)	0.50	-0.1(-0.4,0.1)	0.41	0.87
mCEQ: Enjoyment	-0.3(-0.9,0.3)	0.26	-0.4(-1.0,0.2)	0.23	0.90
mCEQ: Craving	-0.4(-1.0, 0.3)	0.25	-0.4(-1.2, 0.3)	0.26	0.97
CLS 1: Strong	-1.0(-1.8, -0.2)	**0.02**	-1.8(-2.7, -1.0)	**<0.0001**	0.16
CLS 2: Hot	-0.2(-1.0, 0.5)	0.53	-0.3(-1.1, 0.5)	0.43	0.88
CLS 3: Hard to draw	0.06 (-0.6, 0.7)	0.84	-0.5(-1.2, 0.2)	0.19	0.27
CLS 4: Harsh	0.01(-0.8, 0.8)	0.98	-1.6(-2.5, -0.7)	**0.0006**	**0.01**
CLS 5: Taste	-1.6(-2.4, -0.8)	**<0.0001**	-1.3(-2.2, -0.5)	**0.002**	0.66
CLS 6: Satisfying	-1.6(-2.4, -0.8)	**0.0002**	-1.6(-2.5, -0.7)	**0.0004**	0.97
CLS 7: Tobacco vs. air	-0.7(-1.4, -0.02)	**0.04**	-1.6(-2.4, -0.9)	**<0.0001**	0.08
CLS 8: Likelihood to buy	-1.4(-2.4, -0.4)	**0.01**	-1.9(-3.0, -0.9)	**0.0003**	0.47
CLS 9: Nicotine	-0.6(-1.0, -0.3)	**0.0004**	-0.9(-1.3, -0.6)	**<0.0001**	0.23
CLS 10: Hit	-0.5(-0.9, -0.2)	**0.0012**	-0.5(-0.8, -0.1)	**0.01**	0.75

*Treatment effect: VLNC-UNC (differences in means VLNC – UNC for CPD, blood cotinine, exhaled CO, FTCD, HONC, mCEQ and CLS)

[1] Adjusted for baseline measure of the outcomes, study, age, gender, race, education, employment status, and other covariates.

CLS item 1: How strong was the cigarette? (1=not at all, 10=extremely); CLS item 2: How hot was the cigarette? (1=not at all, 10=extremely); CLS item 3: How hard was it to draw? (1=not at all, 10=extremely); CLS item 4: How harsh was the cigarette? (1=not at all,10=extremely); CLS item 5: How much taste did you get from the cigarette? (1=not at all, 10=extremely); CLS item 6: How satisfying was the cigarette? (1=not at all, 10=extremely); CLS item 7: How much tobacco vs. 'just air' did you get from the cigarette? (1=just air, 10=just tobacco); CLS item 8: What is the likelihood that you would buy cigarettes like these? (1=not at all, 10=extremely); CLS item 9: How much nicotine do you think these cigarettes gave you compared to your usual cigarettes? (1=much less, 5=much more); CLS item 10: How satisfying was the hit these cigarettes gave you compared to your usual cigarettes? (1=much less, 5=much more)

Menthol and Smoking Biomarkers of SES and MH Trial

Appendix Table **A-2** shows the treatment effects of VLNC by menthol flavor for each individual study. For SES trial, there were significant reductions in CPD in menthol smokers between the VLNC and UNC groups (difference in CPD: -5.1, 95% CI: -8.8, -1.5; $p<0.01$), The reductions in CPD in non-menthol smokers did not differ significantly between the VLNC and UNC groups (difference in CPD: -3.0, 95% CI: -8.5, 2.5; $p >0.1$). The test for interaction indicated that menthol flavor did not modify the treatment effect of VLNC on CPD reduction ($p_i>0.5$). Significant reductions in blood cotinine levels were found in both menthol and non-menthol smokers ($p<0.001$). The test for interaction of menthol on the treatment effect of VLNC on blood cotinine reduction was significant (-139.7 ng/ml for menthol vs. -262.3 ng/ml for non-menthol; $p_i = 0.02$; Appendix Table **A-2**). Expired CO were lower among the VLNC group than UNC group, but the reductions were not significant among menthol (-3.3 ppm, 95% CI: -8.6,1.9; $p>0.10$) or non-menthol smokers (-2.7 ppm, 95% CI: -10.5,5.1; $p>0.10$). The test for interaction was not significant between nicotine content treatment (VLNC vs. UNC) and cigarette flavor (menthol vs. non-menthol) on expired CO ($p_i>0.5$).

For MH trial, significant reductions in CPD were found in menthol smokers between VLNC and UNC groups (difference in CPD: -8.1, 95% CI: -12.9, -3.2; $p<0.01$; Appendix Table **A-2**), but no significant difference among non-menthol smokers between VLNC and UNC (difference in CPD: -2.8, 95% CI: -6.6, 1.0; $p >0.1$). The test for interaction was not significant ($p_i=0.10$). Significant blood cotinine reductions were observed in both menthol ($p<0.0001$) and non-menthol ($p<0.0001$) cigarette smokers, the treatment effect of VLNC on cotinine reduction was similar (-190.2 ng/ml for menthol vs. -187.2 ng/ml for non-menthol; $p_i>0.5$; Appendix Table **A-2**). Expired

CO were significantly lower among the VLNC group than UNC group among menthol smokers (mean difference: -11.8, 95% CI: -19.5, -4.1; $p<0.05$) and non-menthol smokers (mean difference: -7.0, 95% CI: -12.9, -1.2; $p<0.05$). Interaction term was not significant between nicotine content treatment and cigarette flavor ($p_i>0.1$).

Study Compliance among VLNC Cigarette Users Only

Table **4-7** lists the odds ratio of being compliant by cigarette flavor (Non-menthol vs. Menthol) among VLNC group. For SES trial, non-menthol smokers had higher percentage of being compliant with using only study cigarettes compared to menthol groups (79% vs. 33%; p-value< 0.01). For MH trial, non-menthol smokers had higher percentage of being compliant than menthol smokers, but the difference between these two groups is not significant (68% vs. 54%; p-value: 0.26).

After combining SES and MH trials, menthol smokers presented much lower percentage of compliance compared to non-menthol smokers (41% vs. 70%; p-value< 0.001). The odds ratio for being compliant for nonmenthol vs. menthol users is 2.6 (95% CI:1.0, 6.4; p-value: 0.04) after adjusting baseline age, study, gender, race, education, household income, and employment status (Table **4-7**).

Table **4-7.** Odds Ratio of compliance by cigarette flavor (Non-menthol vs. Menthol) among VLNC only[1]

	Menthol	Non-menthol	p-value
SES			
Compliance, N (%)	16 (33.3)	11 (78.6)	**0.003**
Non-compliance, N (%)	32 (66.7)	3 (21.4)	
MH			
Compliance, N (%)	14 (53.9)	27 (67.5)	0.26
Non-compliance, N (%)	12 (46.1)	13 (32.5)	
Pooled results (SES + MH)			
Compliance, N (%)	30 (40.5)	38 (70.4)	**0.0008**
Non-compliance, N (%)	44 (59.5)	16 (29.6)	
Crude OR (95% CI)	1.00 [Reference]	3.5 (1.7, 7.4)	**0.001**
Adjusted OR (95% CI) [2]	1.00 [Reference]	2.6 (1.0, 6.4)	**0.04**

[1] Study compliance was assessed at the last visit of randomization phase
[2] Logistic regression model, adjusted for baseline age, gender, race, education, household income, employment status, and study.

4.4 Discussion

We found that the effect of switching to VLNC through gradually reduced nicotine was lower in menthol smokers in pooled analysis of randomized trials. Menthol smokers did not achieve the same level of nicotine reduction as nonmenthol smokers. In pooled analysis, menthol was not a significant effect modifier, but did have significant effect modification in the SES trial. There were little differences in reduction in measures of nicotine dependence between smoking menthol and nonmenthol VLNC. Subjective rating is another important way to reflect smokers' acceptability and sensory effects of low nicotine products. [98,99] There was no significant interaction detected in mCEQ subscales and the cigarette liking scale for both trials. These results indicated that smokers made similar subjective rating on the reinforcing effects and taste perceptions to study cigarettes regardless of their menthol status. Menthol did not affect nicotine reduction in the MH trial. There are mixed findings on whether smokers with mental health problems are more likely to prefer menthol cigarettes, depending on age, severity of the illness, and how mental health illness is assessed. [135] Menthol sensation is thought to have stimulating effects perceived favorably by persons with mental illness, but the current findings of a lack of menthol effect on nicotine reduction in the MH trial is more consistent with the idea that nicotine is used to self-regulate mood disorder. [136]

As reported previously, both immediate (Hatsukami et al.)[86] and gradual nicotine reduction to VLNC (Krebs et al.)[90] trials presented significant lower smoking and toxicant exposure than UNC groups. However, demonstrating compliance to using VLNC cigarettes is especially important for informing a nicotine reduction policy because the proposed policy is likely based on setting a maximum nicotine level at very low levels rather than just moderately reduced levels. We found that the odds of being compliant with using only VLNC study

cigarettes was almost 3 times higher in non-menthol than menthol smokers. Menthol smokes are less likely to achieve smoking minimally addicting cigarettes. These findings favor the idea that removing menthol flavor from cigarettes would be beneficial under a proposed nicotine standard.

The focus of our paper on gradual VLNC has several public health implications. Menthol smokers who find VLNC unacceptable may seek to purchase fully nicotinized cigarettes from the black market to avoid withdrawal symptoms.[99] To avoid this, it will be important to find more effective forms or dose of noncombustible nicotine to assist these smokers in transitioning to VLNC and ultimately cessation. Menthol flavoring may be important for the success of transitional nicotine products.

We acknowledge some limitations in our study. First, cigarette flavor status differed by race. Most Black individuals prefer menthol-flavored cigarettes, and the sample size of non-menthol black was low for both trials (n=3 for SES, and n=6 for MH). Therefore, an assumption in the findings is that any outcomes that vary by menthol status is due to flavor itself and not due to race. Second, cigarette smokers chose the flavor of their study cigarettes based on their preference. Thus, cigarette flavor (menthol status) was not randomized, which could introduce bias and distort the relationship between menthol status and VLNC outcomes. [93] Third, neither SES or MH trial measured or compared total nicotine equivalents (TNE), which may serve as a better estimate of tobacco smoke exposure in slow nicotine metabolizers. [137] Fourth, the inclusion criteria for the trial were a minimum 5 cigarettes per day, which may limit inferences on nicotine reduction in infrequent smokers.

There are other reasons for considering banning menthol beyond its potentially lesser impact on nicotine reduction. The tobacco industry has historically marketed different tobacco products, especially menthol cigarettes, to black in urban communities. [125] As a result of the large

amount of advertising placed by tobacco companies, menthol cigarettes has facilitated the onset of smoking and nicotine addiction in Black youths [102] and adults.[138] Thus, the impact of menthol on smoking initiation is also a major consideration.

Chapter 5

Discussion

Tobacco industry has historically marketed different tobacco products, especially menthol cigarettes, to Black Americans in urban communities.[139] As a result of the large amount of advertising placed by tobacco companies, seven out of ten Black youth[102] and more than 85% of Black smokers used menthol cigarettes.[138] Black Americans constituted only 12% of the total US population, but menthol is responsible for 1.5 million extra smokers, more than 150,000 smoking-related premature deaths and 1.5 million excess life-years lost during 1980-2018.[138] On April 29, 2021, FDA announced its commitment to ban menthol as a characterizing flavor in cigarettes and ban all characterizing flavors (including menthol) in cigars within the next year. A menthol ban would have saved up to 633,252 lives and almost one third of them would be blacks from 2011 to 2050 as projected by the smoking simulation model.[140]

The health effects of menthol added to tobacco have been reviewed including a 2013 FDA evaluation of the impact of menthol on public health. [139,141] Since 2017, forms of menthol bans have been implemented at the local level in several states, and in the European Union, Brazil, most of the Canadian Provinces, and other countries. [142] Many aspects of tobacco harm and addiction were reviewed including smoke chemistry, targeted marketing, tobacco use initiation and progression, nicotine dependence and disease risk. One area of tobacco harm that remains inconclusive is whether menthol affects exposure to tobacco smoke constituent biomarkers. Menthol has been studied in relation to the nicotine metabolite cotinine, but there is little information on exposure to tobacco carcinogens. PAHs and VOCs are among the major classes of tobacco carcinogens and toxicants. The availability of more recent biomarker data allows for the determination of the effects of menthol in cigarettes on urine metabolites of PAHs,

VOCs, and heavy metals. In Chapter 3, we analyzed levels of these biomarkers from the special sample of NHANES 2015-2016 by cigarette menthol flavor status. We found that there were no significant differences in 22 of 26 HPHCs by menthol status, providing supporting evidence that menthol in cigarettes does not affect exposure to tobacco smoke constituents. It appears the negative impact of menthol may not be due to increased exposure to carcinogens and toxicants, but rather its effect on the initiation of cigarettes, severity of dependence, and poorer cessation rates observed in menthol smokers. In addition to PAHs and VOCs, it was previously reported in studies of NHANES that exposure to the tobacco-specific nitrosamine NNK, another IARC classified lung carcinogen, [77,78,119] does not appear to differ by menthol status as determined by its urinary biomarker NNAL. [55,56] These findings appear consistent with studies that showed the rates and risk of lung cancer were similar in menthol and non-menthol smokers. [143-148] PAHs in cigarette smoke have also been linked to cardiovascular disease, [149] and the current findings are also consistent with similar risks of CVD between menthol and non-menthol smokers. [150]

We also examined the relationship between menthol and NMR, which is positively associated with cigarette consumption. [151] The NHANES started to provide 3'-hydroxycotinine and cotinine measurements in 2013 and the data on special smoker sample with urinary biomarker concentrations became available for the first time with 2015-2016 wave of the survey. In-vitro studies suggest that menthol may impact metabolism of nicotine through inhibition of CYP2A6 activity [38] and clearance of nicotine, [29] where higher NMR is sometimes associated with greater cigarette consumption. Using the nationally representative sample of smokers, we found that urine NMR did not differ between menthol and non-menthol cigarette smokers.

We are not advocating that menthol cigarettes are in any way "safer" or "safely made" than non-menthol cigarettes. Tobacco smoke contains over thousands of chemicals, including thousands of toxicants that lead to malignancy and disease, regardless of the cigarette flavor being smoked. The results of our study should be considered within the context of several

limitations. Cigarette brands are classified as menthol and non-menthol by their manufacturers, but most cigarettes contain low levels of menthol even if they are classified and/or marketed as nonmenthol brands. [104,120] Also, there may be other cigarette design characteristics besides menthol flavor that can affect the exposure to tobacco smoke constituents. [121] The major public health burden of added menthol is its role in facilitating smoking initiation among youth. [15,20-24] Separately, menthol may also facilitate increased tobacco smoke inhalation, but we did not find direct evidence of this effect in the current study as shown by similar cotinine levels with non-menthol smokers. Notably, the generalizability of our findings may be limited by the exclusion of dual/poly tobacco product users, such as dual users of cigarette and electronic cigarette (e-cigarette) given e-cigarette use has increased significantly worldwide among youth. [152,153] Although the NHANES 2015-2016 included information on e-cigarette use, the sample size for dual users was considered too small to conduct an analysis by menthol use. The NHANES 2015-2016 special sample captures data on adult smokers who smoke cigarettes every day, which hinders the generalizability of the current study results to non-daily smokers.

The concept behind regulating flavor in combustible tobacco products is based on reducing the desirability of that product so that smokers switch to lower harm options including cessation as the most desirable outcome or switching to noncombustible nicotine products. In the current study (Chapter 4), we didn't find any benefits of non-menthol vs menthol cigarettes in terms of reduced tobacco smoke exposure or dependence. Non-menthol and menthol research cigarettes yielded similar results for several outcomes including CPD, nicotine dependence, nicotine symptomology and cigarette taste perceptions. Menthol smokers were significantly less likely to smoke VLNC cigarettes exclusively and supplement VLNC with their usual nicotinized cigarettes. These findings would appear to be supportive of a regulatory menthol ban in combustible cigarettes under a nicotine reduction standard to minimally addicting levels. From a public health perspective that considers the needs of smokers affected by nicotine regulation,

menthol smokers may require assistance in transitioning from menthol cigarettes to quitting, or if still nicotine dependent to lower risk alternative noncombustible nicotine products under a menthol ban.

In addition to compliant with using only study cigarettes, subjective rating to study cigarettes is another important way to reflect smokers' acceptability and sensory effects of low nicotine products. In a previous report of the low SES trial, we found that RNC smokers rated significantly lower scores only in satisfaction and psychological reward, whereas no difference in enjoyment of respiratory tract sensations and craving reduction between RNC and UNC groups.[98] Specifically, visit 6 of SES trial is usually the start point of where there are significant differences for some mCEQ and CLS measures between two treatments. The nicotine content for this visit is 1.4 mg nicotine per cigarette.[98] Thus, visit 6 or 1.4 mg nicotine content may play a vital role in participants' rating in liking scale. In light of SES trial, VLNC usually defined as less than 0.6 mg nicotine per cigarette. It is important for researchers to come up with a possible solution to make a smooth transition from 1.4 mg nicotine content cigarette to VLNC with better compliance and lower rates of participant attrition. The findings from our study revealed that 1.4 mg nicotine content can be the key point and cigarette effects (e.g., satisfaction, craving reduction and likelihood of buying) for future VLNC transition research.[98] Another study conducted by Smith et al. found that immediate reduction in nicotine content led to a significant lower score in four out of five mCEQ subscales (satisfaction, psychological reward, enjoyment of respiratory tract sensations, and craving reduction).[99] In other words, the reduction method (immediate vs. gradual) did impact smokers' subjective rating to study cigarettes especially when rating in enjoyment of respiratory tract sensations and craving reduction. A lower satisfaction score is usually considered as a double-edged sword. On the one hand, smokers who are not satisfied with VLNC cigarettes may quit smoking cigarettes, but on the other, addicted smokers may have increased need for illicit normal nicotine content cigarettes or switch to other high nicotine

content tobacco products. Our current study further explored whether the subjective rating modified by the cigarette flavor. As shown in results section, there was no significant interaction detected in mCEQ subscales for both trials. These results indicated that smokers made similar subjective rating to study cigarettes regardless of their menthol status.

Lowering nicotine content in cigarettes had similar beneficial effects by flavor status, except menthol smokers were less likely to smoke VLNC cigarettes without supplementation with usual cigarettes. The findings provide support for a menthol ban which should be based on removing the desirability of smoking combusted cigarettes. Menthol smokers who cannot quit under a nicotine standard in cigarette may require greater assistance and targeted interventions in transitioning to alternative noncombustible nicotine products. A critical consideration in trials designed to guide policy is cotinine reduction, cigarettes per day (CPD), nicotine dependence, expired carbon monoxide (CO), and study compliance to smoking VLNC in the treatment groups. While lowering nicotine and smoke exposure using RNC is desirable, how smokers respond to smoking VLNC, or non-addicting cigarettes is critical for smoking cessation. In theory, the lack of reinforcing effects from cigarettes with minimal nicotine should facilitate cessation. Compliance to using VLNC study cigarettes is a major objective of RNC trials since only VLNC are considered minimally or not addicting. If smokers are able to smoke VLNC without significant withdrawal symptoms and resorting to smoking their regular cigarettes, their ability to quit smoking altogether is expected to be increased substantially. However, black market high nicotine cigarettes and little cigars might be potential harmful alternatives if VLNC are the only available marketed cigarettes.

In April 2022, FDA proposed product standards to prohibit menthol as a characterizing flavor in cigarettes. The proposed rules would help prevent youths from becoming the next generation of smokers and help adult smokers quit. Furthermore, the proposed rules represent a vital step to advance health equity by reducing tobacco-related health disparities. Tobacco

product standards and regulations are FDA's most powerful and critical tools to achieve its public health mission. The modeling study estimated a 15 percent reduction in smoking within 40 years if menthol cigarettes were no longer available in the United States.[154] Through the rulemaking process, the public can provide comments on these proposed rules. It is an important opportunity for the public to make their voices heard and help shape the FDA's future action. Pending public comments, the FDA will consider issuing a final product standard. These timely actions may benefit public health by significantly reducing disease and death attributed to traditional cigarette smoking.

Chapter 6

Summary and Future Directions

In summary, we tested a few different hypotheses in this dissertation to investigate the impact of menthol cigarette flavor on the nicotine metabolite ratio, biomarkers of tobacco exposure, and very low nicotine content cigarettes. We first conducted the most comprehensive literature review up to date assess the association between menthol flavor and NMR. Then we used a nationally representative sample of US population to further evaluate the potential impact of menthol cigarette.

In chapter 3, we obtained the representative sample of US smokers from the NHANES and calculated adjusted geometric means of urine NMR for menthol and non-menthol cigarette smokers. Also, different biomarkers of tobacco exposure such as nicotine, cotinine, 3-HC, urine metabolites of PAHs, VOCs and metals were compared by menthol status. Menthol cigarette use is not associated with higher exposure to carcinogenic and toxic constituents. Our study results on toxicity are similar for menthol and non-menthol cigarettes.

The findings from Aim 1 do not support our hypothesis that menthol may inhibit nicotine metabolism. Most prior studies investigated the association between menthol and NMR with small sample size. We used large sample size of nationally representative US smokers. Therefore, the study findings from our NHANES 2015-2016 Special Sample may be more impactful than other randomized trials. The findings from Aim 2 do not support our hypothesis that menthol smokers may have higher biomarkers of toxicant exposure. Menthol's cooling sensation in cigarettes may allow smokers to inhale more deeply and result in higher levels of biomarkers of toxicant exposure.[100] However, we found that menthol cigarette use was not associated with higher exposure to carcinogenic and toxic constituents. These findings align with prior studies

that showed the rates and risk of lung cancer were similar in menthol and non-menthol smokers. [143-148] PAHs in cigarette smoke have also been linked to cardiovascular disease, [149] and the current findings are also consistent with similar risks of CVD between menthol and non-menthol smokers. [150]

There were no significant differences in 22 of 26 HPHCs by menthol status, providing supporting evidence that menthol in cigarettes does not affect exposure to tobacco smoke constituents. However, there is considerable data summarized by the FDA that support a menthol ban including its effect on increased experimentation, initiation, and dependence in youths and young adults, particularly in Black smokers. Menthol is also associated with poorer cessation rates. On April 28, 2022, FDA announced proposed product standards to prohibit menthol as a characterizing flavor in cigarettes. These timely actions may benefit public health by significantly reducing disease and death attributed to traditional cigarette smoking.

In chapter 4, we investigated the effect of menthol on very low nicotine content cigarettes through two randomized controlled trials. Both trials were managed under pharmacy-controlled protocols and were conducted to determine the effects of nicotine reduction in populations with high rates of smoking. The PSU-GWU trial included cigarette smokers with low socioeconomic status (SES; less than a college degree), and the PSU-MGH trial included smokers with the mental health (MH) conditions (mood and/or anxiety disorders) as determined by the structured Mini-International Neuropsychiatric Interview. [94,95] We conducted the pooled analysis of these two trials and found that reduction in cotinine when switching to VLNC was greater for non-menthol than menthol smokers (-208 ng/ml vs. -157 ng/ml, pooled p-value interaction =0.15). Menthol did not modify the pooled association between nicotine content treatment (VLNC vs. UNC) and cigarettes per day, expired carbon monoxide levels, nicotine dependence and symptomology. Menthol did not have a differential impact on multiple subjective ratings of VLNC cigarettes, except harshness. The odds ratio for being compliant for nonmenthol vs.

menthol users is 2.6 (95% CI:1.0, 6.4; p-value: 0.04). Lowering nicotine content in cigarettes had similar beneficial effects in menthol and nonmenthol smokers, except menthol smokers were less likely to smoke VLNC cigarettes without supplementation with usual cigarettes. The findings provide support for a menthol ban which should be based on removing the desirability of smoking combusted cigarettes. The public health impact of this dissertation is that menthol smokers who cannot quit under a nicotine standard in cigarette may require greater assistance and targeted interventions in transitioning to alternative noncombustible nicotine products.

The findings from Aim 3 do not support our hypothesis that the magnitude of change in blood cotinine and smoking behavior may be smaller in menthol smokers than non-menthol smokers. Lowering nicotine content in cigarettes had similar beneficial effects by flavor status. Our study findings differ from results of clinical trials testing effects of immediately reduced VLNC cigarettes, where menthol smokers respond to a nicotine reduction policy with smaller reductions in smoking rates and toxicant exposure than non-menthol smokers.[93] SES and MH trials were not originally powered to detect interaction effect of cigarette nicotine content and menthol smoking status. Therefore, in addition to the different methods of nicotine reduction (gradual vs immediate), the limited power may partially explain the inconsistencies between our study findings and results of the immediate VLNC trial.

As reported previously, both immediate [86] and gradual nicotine reduction to VLNC [90] trials presented significant lower smoking and toxicant exposure than UNC groups. However, demonstrating compliance to using VLNC cigarettes might be important for informing a nicotine reduction policy because the proposed policy is likely based on setting a maximum nicotine level at very low levels rather than just moderately reduced levels. We found that the odds of being compliant with using only VLNC study cigarettes for non-menthol smokers was almost 3 times the odds for menthol smokers. Menthol smokers are less likely to achieve smoking minimally addicting cigarettes. This may be in-part related to neurobiological interactions between menthol

and nicotine that result in higher nicotine dependence. [155] These findings favor the idea that removing menthol flavor from cigarettes (menthol ban) would also be beneficial under a possible nicotine standard in the future, where transitioning to very low nicotine cigarettes might be easier if the only cigarette flavor option to smokers available is tobacco flavor. However, menthol smokers who find VLNC unacceptable may seek to purchase fully nicotinized cigarettes from the black market and little cigar products to avoid withdrawal symptoms. [99] To avoid this, it will be important to find more effective forms or dose of noncombustible nicotine to assist these smokers in transitioning to VLNC and ultimately cessation. Menthol flavoring may be important for the success of transitional nicotine products. Overall, our study suggests that if a mandated reduction in nicotine content is implemented, a VLNC standard in cigarettes policy is likely to benefit public health. Nonetheless, nicotine reduction policy might also produce greater interest in illicit cigarettes and black-market high nicotine cigarettes.

Despite the novelty of this dissertation, there are some limitations that need to be addressed. For both SES and MH trials, cigarette smokers chose the flavor of their study cigarettes based on their preference. Thus, cigarette flavor (menthol status) was not randomized, which could introduce bias and distort the relationship between menthol status and VLNC outcomes. [93] Further, our trials did not measure or compare total nicotine equivalents (TNE), which may serve as a better estimate of tobacco smoke exposure in slow nicotine metabolizers. [137] In addition, we were underpowered to test the interaction effects of cigarette nicotine content and menthol status.

The potential health benefits associated with menthol ban and nicotine reduction policy have gained attention from regulatory community. Findings from this dissertation will provide helpful directions for future studies about the potential benefits of menthol ban and VLNC standard policy. However, it is important to note that the association between menthol flavor and biomarkers of toxicant exposure are not conclusive. Furthermore, we had several limitations, such

as the cross-sectional nature of the NHANES survey, lack of data on non-daily cigarette smokers, and lack of data on tobacco specific nitrosamines. There are still a few questions left unanswered, for example, the long-term association between menthol cigarette smokers and carcinogen biomarkers, the impact of menthol flavor on smoking behaviors and nicotine dependence in dual users of cigarette and electronic cigarette (e-cigarette), and the impact of flavored little cigars on HPHCs exposure. Larger prospective cohort studies are warranted to validate the potential health benefits of menthol ban. Compliance to using VLNC study cigarettes is critical for the nicotine reduction policy. Findings from our study may not reflect the real-world situation since smokers will be aware of the reduced nicotine content from the cigarette package if the FDA decides to implement a reduced nicotine policy. Further, our trials were conducted in a pre-policy environment where participant's usual brand cigarettes were still available, providing the opportunity for non-compliance with only smoking the research cigarettes. Compliance to using VLNC study cigarettes would facilitate the goal of nicotine reduction policy, but also points to the need to reduce access to black market cigarettes. Future epidemiological studies are warranted to investigate the effects of VLNC on smoking behavior and product satisfaction if a mandated reduction in nicotine content is implemented.

Appendix

Table A-1. VOC metabolites and their parent compounds

Parent Compound	Common Name	Analyte Name
Xylene	2-MHA	2-Methylhippuric acid
Xylene	3-MHA and 4-MHA	3- and 4-Methylhippuric acid
Acrylamide	AAMA	N-Acetyl-S-(2-carbamoylethyl)-L-cysteine
N, N-Dimethylformamide	AMCC	N-Acetyl-S-(N-methylcarbamoyl)-L-cysteine
Cyanide	ATCA	2-Aminothiazoline-4-carboxylic acid
Toluene	BMA	N-Acetyl-S-(benzyl)-L-cysteine
1-Bromopropane	BPMA	N-Acetyl-S-(n-propyl)-L-cysteine
Acrolein	CEMA	N-Acetyl-S-(2-carboxyethyl)-L-cysteine
Acrolein	3HPMA	N-Acetyl-S-(3-hydroxypropyl)-L-cysteine
Acrylonitrile	CYMA	N-Acetyl-S-(2-cyanoethyl)-L-cysteine
1,3-Butadiene	DHBMA	N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine
Propylene oxide	2HPMA	N-Acetyl-S-(2-hydroxypropyl)-L-cysteine
Styrene	MA	Mandelic acid
Ethylbenzene, styrene	PGA	Phenylglyoxylic acid
Crotonaldehyde	HPMMA	N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine

Table A-2. Treatment Effects on Outcomes (SES and MH Trials)

	SES					MH				
Outcome[1]	Menthol smokers		Non-menthol smokers		Interaction	Menthol smokers		Non-menthol smokers		Interaction
	Treatment effect*	p-value	Treatment effect*	p-value	p-value	Treatment effect*	p-value	Treatment effect*	p-value	p-value
CPD	-5.1(-8.8, -1.5)	**0.0065**	-3.0(-8.5, 2.5)	0.27	0.52	-8.1(-12.9, -3.2)	**0.0012**	-2.8(-6.6, 1.0)	0.15	0.10
Cotinine	-139.7 (-198.6, -80.9)	**<0.0001**	- 262.3(-347.9, -176.7)	**<0.0001**	**0.0192**	-190.2(-272.8, -107.6)	**<0.0001**	-187.2(-249.8, -124.5)	**<0.0001**	0.95
Exhaled CO	-3.3(-8.6, 1.9)	0.20	-2.7(-10.5, 5.1)	0.49	0.89	-11.8(-19.5, -4.1)	**0.0031**	-7.0(-12.9, -1.2)	**0.0195**	0.34
mCEQ: Satisfaction	-0.6(-1.2, 0.1)	0.08	-1.0(-2.0, -0.1)	**0.03**	0.39	-1.4(-2.4, -0.5)	**0.0044**	-0.6(-1.4, 0.1)	0.1	0.21
mCEQ: Reward	-0.4(-0.9, 0.1)	0.08	-0.4(-1.1, 0.2)	0.20	0.92	-0.8(-1.6, -0.02)	**0.04**	-0.7(-1.4, -0.1)	**0.02**	0.84
mCEQ: Aversion	-0.2(-0.5, 0.2)	0.29	0.07(-0.4, 0.6)	0.78	0.41	0.2(-0.2, 0.5)	0.41	-0.2(-0.5, 0.1)	0.20	0.17
mCEQ: Enjoyment	-0.1(-0.8, 0.7)	0.89	-0.5(-1.6, 0.6)	0.35	0.49	-1.2(-2.3, -0.2)	**0.03**	-0.2(-1.0, 0.7)	0.68	0.14
mCEQ: Craving	0.02(-0.8, 0.9)	0.94	-1.3(-2.6, -0.08)	**0.04**	0.07	-1.2(-2.4, -0.02)	**0.04**	-0.1(-1.0, 0.9)	0.90	0.15
CLS 1: Strong	-0.8(-1.9, 0.3)	0.13	-2.2(-3.8, -0.6)	**0.0070**	0.15	-1.6(-2.9, -0.3)	**0.0197**	-1.8(-2.8, -0.8)	**0.0010**	0.83
CLS 2: Hot	-0.9(-1.9, 0.1)	0.08	-0.6(-2.0, 0.9)	0.46	0.71	0.6(-0.8, 2.0)	0.36	-0.3(-1.3, 0.8)	0.65	0.33
CLS 3: Hard to draw	0.4(-0.4, 1.2)	0.36	-1.4(-2.5, -0.2)	**0.03**	**0.0186**	-0.5(-1.7, 0.8)	0.44	-0.08(-1.0, 0.9)	0.86	0.62
CLS 4: Harsh	-0.1(-1.3, 1.0)	0.80	-1.7(-3.4, -0.003)	**0.05**	0.14	0.3(-1.2, 1.8)	0.68	-1.6(-2.8, -0.5)	**0.0059**	0.05
CLS 5: Taste	-2.5 (-3.4, -1.6)	**<0.0001**	-1.2(-2.6, 0.1)	0.08	0.12	-0.7(-2.1, 0.8)	0.37	-1.4(-2.5, -0.3)	**0.0156**	0.44
CLS 6: Satisfying	-1.6(-2.5, -0.6)	**0.0018**	-1.3(-2.8, 0.1)	0.07	0.78	-1.9(-3.6, -0.4)	**0.0140**	-1.8(-3.0, -0.6)	**0.0045**	0.86
CLS 7: Tobacco vs. air	-1.1(-1.9, -0.3)	**0.0097**	-0.7(-1.9, 0.6)	0.30	0.55	-0.7(-1.9, 0.6)	0.27	-2.0(-3.0, -1.0)	**0.0001**	0.12
CLS 8: Likelihood to buy	-1.7(-2.9, -0.5)	**0.0046**	-2.2(-3.9, -0.5)	**0.0127**	0.66	-1.3(-3.1, 0.6)	0.17	-1.6(-3.0, -0.2)	**0.0258**	0.78
CLS 9: Nicotine	-0.9(-1.3, -0.4)	**0.0002**	-1.0(-1.6, -0.3)	**0.0043**	0.83	-0.4(-1.0, 0.2)	0.21	-0.9(-1.3, -0.4)	**0.0004**	0.24
CLS 10: Hit	-0.6(-0.9, -0.1)	**0.0110**	-0.03(-0.7, 0.6)	0.93	0.16	-0.6(-1.2, 0.02)	0.06	-0.7(-1.1, -0.2)	**0.0047**	0.81

*Treatment effect: VLNC-UNC (differences in means VLNC – UNC for CPD, blood cotinine, exhaled CO, mCEQ and CLS)

[1]Adjusted for baseline measure of the outcomes, age, gender, race, education, employment status, and other covariates.

Bibilography

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Selected Publications

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Lin, Wenxue, and Joshua E. Muscat. "Knowledge and Beliefs Regarding Harm From Specific Tobacco Products: Findings From the HINT Survey." *American Journal of Health Promotion* (2021): 08901171211026116.PMID: 34338002.

Joshua E. Muscat, **Lin, Wenxue**. Global Perspective on Understanding the Potential Benefits and Risks of E-Cigarette use on Head and Neck Cancer. *J Oral Cancer Res* (2020) 3(1):28-30

Lin, Wenxue, Sydney A. Martinez, Kai Ding, and Laura A. Beebe. "Knowledge and Perceptions of Tobacco-Related Harm Associated with Intention to Quit among Cigarette Smokers, e-Cigarette Users, and Dual Users: Findings from the US Population Assessment of Tobacco and Health (PATH) Wave 1." *Substance Use & Misuse* (2021): 1-7. PMID: 33594931

Goyal, Neerav, Max Hennessy, Erik Lehman, **Wenxue Lin**, Antonio Agudo, Wolfgang Ahrens, Stefania Boccia et al. "Risk factors for head and neck cancer in more and less developed countries: Analysis from the INHANCE Consortium." *Oral Diseases* (2022). PMID: 35322907

Awards and Honors

Alumni Society Award, College of Medicine, Penn State University. — Nov. 2021