1. Principal Investigator: Mehmet Sofuoglu, M.D., Ph.D.

2. Co-Investigators (including VA and WOC staff): While all research staff are trained to provide informed consent, those identified with an asterisk (*) will provide informed consent for this study.

Co-Investigators: Joel Gelernter, M.D., *Gerald Valentine, M.D.
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Research Staff: *Stacy Minnix, BSW, *Lance Barnes, and *Chris Cryan, * Michael Stephens

3. Title of the Project

Effects of Flavors on Nicotine Reinforcement in Smokers

4. Purpose, Hypothesis and Key Questions

Specific Aim #1: To examine the acute reinforcing effects of menthol, a fruit flavor (green apple) or a fruit flavor plus menthol, alone or in combination with nicotine in smokers. Flavors will be administered by inhalation via electronic cigarettes (e-cigarettes) and nicotine will be administered intravenously. The reinforcing drug effects will be measured with the drug effects questionnaire (DEQ).

Hypothesis: Hypothesis #1A: Combined fruit flavor (green apple) plus menthol will be more reinforcing than menthol alone or fruit flavor alone.

Hypothesis #1B: Fruit flavor plus menthol will be more effective than menthol alone, or fruit flavor alone, in enhancing the reinforcing effects of nicotine.

Exploratory Aims: To examine if fruit flavor plus menthol will be more effective than menthol or fruit flavor alone in enhancing nicotine's effects in a) alleviating urges to smoke, b) suppressing withdrawal severity and c) enhancing cognitive performance.

5-6. Background and Significance:

a) Role of Flavors in Tobacco Addiction

With the exception of menthol, all characterizing flavors have been banned from use in combustible tobacco cigarettes. However, flavors are not prohibited from use in other tobacco product categories where the majority, including e-cigarettes, cigars and cigarillos, contain a wide variety of flavorants. Prior studies with tobacco cigarettes have shown that the addition of sweet flavors increased the appeal of these products, especially among youth (King et al. 2014). Similar recent data for non-combustible tobacco products has informed the U.S. Food and Drug Administration's (FDA) prioritization of understanding the impact of flavors on the appeal and use of flavored tobacco products.

Flavors are particularly ubiquitous in e-cigarettes and refill liquids marketed for use in the expanding array of electronic nicotine delivery systems (ENDS). A recent study estimated that there are over 7,000 unique e-cigarette flavors including menthol/mint, fruit, desert/sweet,

tobacco, coffee/tea, nuts/spices and alcohol flavors (Zhu et al. 2014). Many survey and epidemiological studies found that e-cigarette users, especially youth, prefer e-liquids with flavors (Pesko et al. 2016; Soule et al. 2016). For example, in one survey of high school students who use e-cigarettes, over 70% reported having experience with flavored e-liquids and over 50% prefer using e-liquids with sweet flavors (Krishnan-Sarin et al. 2015). In another study with youth from Texas, 95 to 98% reported using flavored e-cigarette as their first e-cigarette, compared to 44 % of older adults in nationwide sample (Harrell et al. 2017). The most commonly used flavors were fruit and sweet flavors and overall, flavors were reported to be a common reason for use of e-cigarettes by youth (Harrell et al. 2017). These studies suggest that flavors increase the appeal of e-cigarettes and may therefore contribute to initiation and maintenance of e-cigarette use.

b) Menthol as a Common Flavor

Menthol is one of the most intensively studied flavors used in tobacco products. It has a well-characterized cooling and soothing action in the airways that may enhance the appeal of menthol cigarettes by reducing the harshness of tobacco smoke (Wise et al. 2012). This action may be particularly significant for youth who are experimenting with combustible tobacco products. For example, in a National Survey on Drug Use and Health, 44.7% of 12 to 17 year-olds smoked menthol cigarettes, compared to 30.1% of adults aged 26 or older (Rock et al. 2010). In addition, numerous cross-sectional studies have shown that menthol cigarette smoking is a risk factor for the development of dependence (Collins and Moolchan 2006; Hersey et al. 2006; Muscat et al. 2009; Wackowski and Delnevo 2007), and a prospective study of smokers aged 17 or younger demonstrated that smoking initiation with menthol cigarettes was associated with higher risk of progression to established smoking, as well as higher levels of nicotine dependence (Nonnemaker et al. 2012). While these studies indicate that menthol plays a role in both the initiation and maintenance of tobacco product use, the underlying mechanisms by which menthol may facilitate nicotine dependence in humans have not been fully elucidated.

In a recent human laboratory study, we examined potential mechanisms underlying menthol's effects in relation to tobacco addiction (Valentine et al. 2017). We found that menthol, administered by inhalation via e-cigarettes, significantly reduced urges to smoke in menthol-preferring, but not in non-menthol-preferring smokers, suggesting that menthol may have negative reinforcing effects by alleviating withdrawal. In addition, menthol-preferring smokers reported diminished positive subjective responses to IV nicotine and less severe tobacco withdrawal than did non-menthol cigarette smokers. These findings are consistent with menthol's effects in reducing the rate of nicotine metabolism or clearance, as indicated by a lower nicotine metabolite ratio, a biomarker for the rate of nicotine metabolism, in menthol-preferring smokers (Benowitz et al. 2004; Fagan et al. 2016). These findings support previous data indicating that one long-term adaptation to menthol exposure may be slowing of nicotine metabolism (DeVito et al. 2016; Sofuoglu et al. 2012). In contrast to such findings for menthol, very little is known about the behavioral pharmacology of flavors or their effects on nicotine metabolism.

c) Research Need for Understanding the Role of Flavors in Tobacco Addiction

The identification of common mechanisms by which flavors may influence tobacco use and appeal is complicated by the availability of thousands of flavor combinations in tobacco products, particularly in e-liquids. To reduce the complexity of research on flavored tobacco products, there have been attempts to categorize e-liquid flavors. For example, one recent survey

suggested that the majority of e-liquid flavors used by consumers can be categorized as either tobacco (23.7%), fruit (20.3%), dessert/sweets (20.7%) or menthol/mint (14.8%) varieties (Yingst et al. 2017). However, as compared to menthol, there is very limited information about the behavioral effects of these other flavor categories. It is important to note that most e-liquid flavors were developed as food additives (e.g., watermelon, apple, cherry, cotton candy or bubble gum), but have not been examined for inhalation use (Tierney et al. 2016). For example, it is unknown if menthol is uniquely different than other flavors that have been used in tobacco products or if flavors have similar effects as menthol. Although many commercially available e-liquid products include a combination of different flavors (e.g., menthol plus fruit or sweet flavors), it is unknown if different flavors have additive or synergistic effects as reinforcers.

Proposed Study

In this study, we will compare the effects of flavored e-liquids that contain menthol alone, a fruit flavor (green apple), and a fruit flavor plus menthol, either alone or in combination with intravenously administered nicotine. Study outcomes will include aversive and positive subjective effects, withdrawal severity, urges to smoke, cognitive performance and cardiovascular measures. We will enroll only menthol-preferring smokers because in our recent study on the acute effects of inhaled menthol, effects were more prominent in menthol-preferring smokers compared to the non-menthol-preferring sample. We chose to use green apple because it represents one of the most commonly consumed e-liquid flavors. The use of menthol, green apple and green apple plus menthol flavors will allow the effects of menthol and fruit flavor to be compared in a systematic way.

7. Subjects:

Young adult male and female smokers will be recruited from the New Haven area through newspaper advertisements, radio advertisements, and fliers. Interested subjects will have the study described over the telephone, and they will be asked to answer a brief tobacco use history and medical screening questionnaire. If subjects pass the telephone screening, they will be invited to come to the West Haven VA clinic for a screening evaluation.

Inclusion criteria: 1) Female and male mentholated cigarette smokers, aged 18 to 30 years; 2) history of smoking for the past 12 months, at least one cigarette per day; smoking status is verified with urinary cotinine levels above 10 ng/ml; 3) not seeking treatment for nicotine dependence at the time of study entry; 4) in good health as verified by medical history, screening examination, and screening laboratory tests; 5) for women, not pregnant as determined by pregnancy screening, nor breast feeding, and using acceptable birth control methods.

Exclusion criteria: 1) History of major medical illnesses that the physician investigator deems as contraindicated for the patient to be in the study; 2) regular use of psychotropic medication (antidepressants, antipsychotics, or anxiolytics); 3) psychiatric diagnosis and / or treatment for Axis I disorders including major depression, bipolar affective disorder, schizophrenia or panic disorder in the past month; 4) abuse of alcohol or any other recreational or prescription drugs in the past 30 days; 5) any allergy to propylene glycol or menthol; and 6) aversion to green apple flavor.

8. Privacy: Participation in research may involve a loss of privacy. To prevent this, research records will be kept as confidential as possible. Only a code number will identify the individual

research records. The code number will not be based on any information that could be used to identify the subject (for example, social security number, initials, birth date, etc.) The master list linking names to code numbers will be kept separately from the research data. All research information will be kept in locked files at all times. Subject identity will not be revealed in any reports or publications resulting from this study. Only authorized research staff will have access to the information gathered in this study.

While filling out study forms, or during the psychiatric examination, some participants may feel uncomfortable disclosing personal thoughts and feelings. Careful efforts aimed at maintaining confidentiality have been effective in previous research, and only participants' code numbers will be recorded on the forms themselves to protect confidentiality.

9. Selection: Interested subjects will have the study briefly described over the telephone, and if interested, they will be asked to answer a brief tobacco use history and medical screening questionnaire. If subjects are eligible for the study based on the telephone screening, they will be invited to come to the clinic for a screening evaluation. The screening evaluation will include the following: a) obtaining informed consent; b) smoking history and assessment of nicotine dependence using the Fagerstrom Test of Nicotine Dependence (FTND) c) complete physical and psychiatric examination including the structured clinical interview (SCID) for DSM-IV; d) laboratory examination including complete blood count, liver and kidney function tests, and glucose; e) urine analysis, drug screen, and for women, urine pregnancy test and f) urine cotinine and urine menthol glucuronide levels.

10. Recruitment: A total of 50 smokers will be recruited from the New Haven area by newspaper advertisements and fliers.

11. Research Plan

A. Overview

We propose a double-blind, crossover study that enrolls young adult smokers who prefer menthol cigarettes. The study will consist of an adaptation session and three test sessions. In the adaptation session, participants will practice using the e-cigarette by sampling the flavors to be used in the test sessions. The test sessions will be performed following overnight abstinence from tobacco. Across the three test sessions, participants will be assigned to a random sequence of the three different e-cigarette conditions: menthol, green apple or menthol plus green apple, a different flavor condition for each test session. In each test session, just after the assigned flavor is delivered via the e-cigarette, participants will receive a random order of one intravenous delivery of saline, and two intravenous deliveries of nicotine (3.6 mcg/kg and 7 mcg/kg or 0.25 mg/70 kg and 0.5 mg/70kg), one hour apart. The test sessions will be performed at least 24 hours apart to minimize carryover nicotine effects. The main outcome measure will be subjective drug effects as measured with the Drug Effects Questionnaire (DEQ). Other outcomes include cardiovascular measures, cognitive performance, and self-report measures of nicotine withdrawal and craving. Cardiovascular measures include heart rate, systolic and diastolic blood pressure. Cognitive performance will be assessed with the Stroop test, mathematical processing test (MPT), and continuous performance test (CPT). Nicotine withdrawal measures will be measured with the Minnesota Nicotine Withdrawal Symptom Checklist (M-NWSC) and the Brief

Questionnaire on Smoking Urges (BQSU).

B. Medical Monitoring and Safety

Subjects will be given a thorough physical examination prior to study entry and a physician will be present at all test sessions. Subjects will be attached to blood pressure and heart rate monitoring devices throughout each test session and an IV catheter will remain in place until the end of each test session. Subjects will be administered nicotine only if the systolic blood pressure is <150 mmHg and heart rate <100 beats/minute. The subject's participation will be terminated if their blood pressure at any time is >170/110 mm Hg, the heart rate is >120 beats/min, or if the subjects develop signs and symptoms compatible with nicotine toxicity. Subjects will be monitored for two hours after the last nicotine administration.

C. Measures

a) Physiological:

- Heart rate and blood pressure: Heart rate and blood pressure readings will be taken at screening, and throughout each test session to measure nicotine's effect on these parameters.

b) Biochemical:

- Serum estradiol and progesterone analysis (females only): Serum estradiol and progesterone levels will be measured before each test session, and will be used as covariates in our analysis because female sex hormones may contribute to sex differences in nicotine responses (Lynch *et al*, 2010).
- Plasma cotinine and 3-hyroxycotinine (3HC): Plasma cotinine and 3-hydroxycotinine levels will be measured from blood samples obtained during the three test sessions to determine the nicotine metabolite ratio (NMR). The NMR is the ratio of 3HC (the primary metabolite of cotinine) to cotinine, and reflects the activity of cytochrome P450 (CYP) 2A6 and therefore, the rate of nicotine clearance (Dempsey *et al*, 2004). NMR has been shown to be stable in smokers during ad-lib and reduced smoking (Mooney *et al*, 2008). Plasma samples for 3HC will be obtained at baseline, and the NMR will be included as a covariate in our analyses.
- Plasma nicotine: Plasma nicotine levels will be measured from blood that was obtained prior to the first experimental vaping session on each test day to characterize the subject's smoking status.
- Urinary cotinine levels: A semi-quantitative rapid urine level of cotinine will be obtained at screening to confirm smoking status at study entry (NicAlert).
- Plasma menthol glucuronide levels: Blood samples collected during each test session will also be used to determine the amount of menthol delivered by the e-cigarette. Samples will be obtained just prior to each of the three experimental vaping sessions (baseline), at 2 time points after each nicotine or saline infusion, and just prior to removing the IV at the end of the study. The plasma levels of menthol glucuronide measured at different time points will be used to generate concentration-time curves for each menthol delivery to characterize the pharmacokinetics of menthol, and to account for differing menthol exposure between subjects.

- Alveolar carbon monoxide: CO readings ≤ 10ppm will be used to verify overnight abstinence from smoking as recommended by the SRNT Subcommittee on Biochemical Verification (Benowitz *et al*, 2002).

c) Subjective:

Measures of Dependence:

- DSM-5 tobacco use disorder items.
- Structured Clinical Interview for DSM-5 (SCID) for Axis I disorders: SCID is a semi-structured interview based on DSM-IV (American Psychiatric Association, 1994) and will be performed to diagnose Axis I psychiatric disorders.
- Fagerstrom Test of Nicotine Dependence (FTND): This self-report measure assesses the degree of nicotine dependence and has been used widely in smoking studies (Heatherton *et al*, 1991).

Measures of Affective State:

- Center for Epidemiologic Studies Depression (CES-D) scale: The CES-D is a 20-item self-report measure of depressive symptoms (Radloff, 1977). This scale will be used at intake to control for baseline differences in depressive symptoms.
- Positive and Negative Affect Schedule (PANAS): The PANAS is a 20-item scale that assesses both negative and positive affective states (Watson *et al*, 1988). This scale is sensitive to the affective symptoms of tobacco withdrawal and predicts relapse to smoking (Kenford *et al*, 2002).

Measures of Acute Drug Effects:

Subjective Drug Effects:

- Drug Effects Questionnaire (DEQ), adapted from Soria (1996), will be used during each test session to measure acute drug responses including: liking, cooling, irritation, strength, feeling, stimulation, good effects, bad effects, head rush, and want more. Each item is rated on a 100 mm scale, from 'not at all' to 'extremely'.

Measures of withdrawal and negative affect:

- Minnesota Nicotine Withdrawal Symptom Checklist (M-NWSC): Smokers will be asked to rate several nicotine withdrawal symptoms on a 100 mm scale, from 'not at all' to 'extremely'. The items are derived from the M-NWSC (Hughes and Hatsukami, 1986) and have been used in previous human laboratory studies (Eissenberg *et al*, 1996). The items include cigarette craving, irritability/anger, anxiety/tension, difficulty concentrating, restlessness, increased appetite, depressed mood, and insomnia.
- Brief Questionnaire on Smoking Urges (BQSU): This 10-item scale, originally developed by Tiffany and Drobes (Tiffany and Drobes, 1991), has been found to be highly reliable and reflects levels of nicotine deprivation (Bell *et al*, 1999; Morgan *et al*, 1999). This scale will be used to measure changes in cigarette craving.

d) Measures of Cognitive Performance:

Cognitive performance will be assessed with 3 tests from the ANAM battery (University of Oklahoma): the Stroop test, the mathematical processing test (MPT), and the continuous performance test (CPT). These 3 tests were chosen because of their sensitivity to tobacco withdrawal and nicotine administration (Heishman *et al*, 2010; Mancuso *et al*, 1999; Myers *et al*, 2008).

- Stroop Test: This test assesses processing speed, selective attention, and interference (Spreen, 1991). It is a computerized version of the classical Stroop Test (Reeves, 2002). The Stroop design consists of one three-minute block of congruent stimuli, and one three-minute block of incongruent stimuli.
- Mathematical Processing Test (MPT): This test assesses basic computational skills, concentration, and working memory. An arithmetic problem involving three single-digit numbers and two operators is displayed (e.g., "5 - 2 + 3 ="). The subject presses buttons to indicate whether the answer to the problem is less than five or greater than five.
- Continuous Performance Test (CPT): This test assesses sustained attention, concentration, and working memory. A target character is displayed for memorization. Then, as individual characters are displayed in sequence, the user presses a designated button only when the displayed character is the target letter.

e) Other Measures:

- E-cigarette Questionnaire: This 10-item self-report questionnaire was developed by our research group to characterize the study populations' experience with, and attitude about, e-cigarettes. It will also include a response item that will identify aversion to green apple flavor. This questionnaire will be administered during the adaptation session.
- Drug Use History: Self-reports of cigarette smoking and use of other tobacco products, alcohol and drugs (cocaine, alcohol, opioids, marijuana, and other illicit drugs) will be assessed with Time Line Follow-Back, which has been shown to be a reliable and valid method for monitoring substance use and other outcomes in longitudinal studies (Miller and DelBoca, 1994; Sobell and Sobell, 1992). The Time Line Follow-Back will be administered at intake.
- Adverse events: SAFTEE: In order to monitor adverse events from the study medications, the SAFTEE will be administered before and after each session. The SAFTEE has been used in a number of pharmacotherapy trials (Levine and Schooler, 1986).

f) Genetic Markers:

We recognize the value of banking biological samples for future genetic, epigenetic and biomarker analyses given the care taken to establish clinical phenotypes. For example, results from genetic studies may confirm the importance of certain TRP-ion channels, or sweet taste receptors, in the effects of menthol and sweeteners on initiation and maintenance of nicotine addiction. These findings would provide a rationale for examining established and novel genetic variants of TRP-channels, and sweet taste receptors, as determinants of responses to flavors in the laboratory. Whenever specific hypotheses are generated in the future that require targeted genetic analysis, this protocol will be amended

to describe the purpose and use of such analyses.

D. Drugs

a) Nicotine: Nicotine bitartrate will be obtained from Interchem Corporation, Paramus, NJ and nicotine solutions for intravenous injection will be prepared by U.S. Specialty Formulations, Bethlehem, PA. On the morning of each test session, syringes containing 5 ml of each infusate will be prepared by the West Haven VA research pharmacy. Common side effects of nicotine include nausea, vomiting, heartburn, elevated heart rate and increased blood pressure.

- IV Nicotine Administration: Nicotine will be administered over 30 seconds using a syringe that is attached to an IV catheter located in a forearm vein. We have followed these procedures in our previous studies (Sofuoglu et al. 2005, 2006b; 2008a, 2009a,b,c) which were completed without any serious and unexpected adverse effects attributed to nicotine, or with other safety concerns. After the last dose, the subjects will stay in the lab for at least two hours, which has been shown to be sufficient for the nicotine-induced changes in heart rate, blood pressure, and subjective effects to return to baseline (Sofuoglu *et al*, 2005; Sofuoglu *et al*, 2006).

- Justification for the nicotine doses: For this proposal, we chose two dose sequences of nicotine (3.6 mcg/kg and 7 mcg/kg or 0.25mg/70 kg and 0.5mg/70kg) with a maximum dose of 0.3 mg and 0.6 mg, respectively, regardless of body weight. In our previous studies, these nicotine doses produced robust subjective and physiological responses (Sofuoglu *et al*, 2009a, Valentine *et al*, 2017). However, the doses used in this study are much lower than doses used in previous studies with addicted smokers (up to 3 mg per injection). Therefore, we believe that the doses used in this study represent an appropriate balance between being sufficiently high to answer the research questions, but low enough to reduce risks to the study participants.

E. Electronic Cigarette

a) Hardware: A Joyetech eGo, tank-type e-cigarette with a single coil atomizer (2.2 ohm) operating at 3.7V (6.2W) will be used as in our previous studies. The e-cigarette will be fitted with a 2 ml plastic disposable tank that allows for loading of the assigned e-liquids into the e-cigarette just prior to each test session.

b) Flavors: Three different flavored e-liquids: 1) *menthol*, 2) *green apple*, and 3) *green apple chill* will be obtained from the AmericaneLiquidStore™, a large internet-based vendor that prepares its products in Wauwatosa, Wisconsin. They claim to be the first e-liquid manufacturer in the United States to obtain International Organization for Standardization (ISO) 9001:2008 and Current Good Manufacturing practices (cGMP) certification. As is common for many large vendors, consumers are able to specify the nicotine level, base liquid mixture and bottle size for each e-liquid product. For this experiment, we will specify e-liquids that are nicotine-free (0.0% nicotine) in a 50%: 50% base mixture of propylene glycol (PG) and vegetable glycerin (VG). The West Haven VA research pharmacy will prepare sealed, 2 ml aliquots of each e-liquid from the stock 132 ml bottles, and store them in a cool dark location until the morning of each test session. These procedures have been successfully deployed in 3 prior e-cigarette studies.

c) Ingredients:

Propylene glycol (USP). PG is the original base liquid for which most e-cigarettes were designed and remains the most common ingredient in base liquids. PG is also credited with producing a throat sensation ('throat hit') that mimics the feel of smoking a cigarette.

Vegetable glycerin (USP). VG is commonly included in e-liquid bases to enhance the volume of vapor production, giving a greater sensory illusion of smoking.

Menthol: Widely used in a range of healthcare products and classified by the FDA as compound that is Generally Recognized as Safe (GRAS), menthol has mild anesthetic, antiseptic and counter irritant properties and is safe for oral ingestion, topical administration and inhalation.

Justification for menthol doses: The menthol present in the two menthol-containing e-liquids is marketed to consumers who prefer light menthol impact. The concentration of menthol in these e-liquids will not exceed 2.0% (20 mg/ml), levels well below those associated with human toxicity. The concentrations of menthol in the stock solutions procured from the AmericaneLiquidStore™ will be verified using an assay developed by Dr. Peter Jatlow.

Green apple flavorant: This proprietary fruit flavor is composed of simple esters and aldehydes dissolved in the base liquid for a final flavor concentration of approximately 2% (net wt. %). Green apple flavorants are commonly used chemicals used in a wide array of food and cosmetic products. Although rare, allergic reactions to green apple flavorants can occur, and any subject reporting intolerance to products with green apple flavor or aroma will be excluded from study participation. Green apple flavor constituents are classified by the FDA as GRAS.

Justification for green apple flavor: Although thousands of different flavor combinations are currently available to e-cigarette consumers, current evidence indicates that second to tobacco flavors, fruit flavors are the most commonly used flavor category in e-liquids upon initiation of e-cigarette use (Farsalinos et al. 2013). Among fruit flavors, green apple flavor is identified by the AmericaneLiquidStore™ as one of their best selling products (also confirmed by personal communication from Dr. Tony Pace, PEC, Wisconsin).

F. Genotyping

DNA Extraction and Genotyping:

DNA will be extracted from peripheral blood using a commercial kit (Paxgene™; PreAnalytiX Qiagen/BD, Germany). We will genotype variations that may modulate nicotine responses using a 384-well plate format. Briefly, DNA samples will be genotyped in 2 µl-reactions. Each reaction will be prepared with 1 ng genomic DNA, 0.05 µl of 20× (or 0.025 µl of 40×) minor groove binder (MGB) probes and primers (hCV8950074 and hCV26000428) (supplied as pre-validated SNP Assays on Demand, Applied Biosystems) 1 µl of 2× TaqMan Universal PCR Master Mix, and 0.004 µl of 100× bovine serum albumin (New England Biolabs). Reactions will be genotyped for an initial denaturation at 95°C for 10 min followed by 60 cycles of 92°C for 15 s and 60°C for 1 min. Discrimination will be performed using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). All

samples will be genotyped in duplicate. We will also examine messenger RNA (mRNA) levels extracted from the peripheral blood. We are especially interested to examine the changes in mRNA levels related to menthol's effects (TRP-ion channels) and to the effects of nicotine. About 2.5 ml of blood will be drawn into the PAXgene tubes at baseline, prior to each IV infusion, and at the end of the session. Extracted mRNA samples will be stored at -80°C in the Genetics Laboratory.

G. Study Procedures

For 24 hours prior to each test session, subjects will be asked to avoid using products containing menthol such as mint teas, cough lozenges, and mint-flavored gum. A trial-size tube of toothpaste that does not contain menthol will be provided for use the morning of each test session. Subjects will be required to abstain from smoking for 10 hours. Abstinence will be verified by expired air CO levels of < 10 ppm (Benowitz et al. 2002) measured on the morning of testing. Subjects will also be asked to refrain from consuming alcoholic beverages and drugs during study participation. Abstinence will be verified by urine drug screening and Breathalyzer measurements before the test sessions. If results indicate non-compliance with these study procedures, the session will be rescheduled. Repeatedly non-compliant subjects will be discharged from the study. Subjects will be instructed to drink their typical amount of caffeinated beverages in the morning to minimize caffeine withdrawal, which could confound interpretation of study measures. Subjects will be given a light breakfast before each test session and a lunch will be provided at the end of each test session.

a) Adaptation Session: The goal of this session is to familiarize participants with the study procedures including the use of e-cigarettes, and to gather information regarding participants' use and knowledge about e-cigarettes. This session will also allow for a determination of the participants tolerance of, or aversion to, any of the flavors prior to participation in a test session. Participants will be given an e-cigarette and instructed to inhale more softly, but for a longer duration (3-4 seconds), than is customary for a standard cigarette. Additionally, a two-phase inhalation is suggested where the vapor from this elongated inhalation is initially gathered in the mouth (as one might do with pipe tobacco) but then further inhaled into the lungs. The e-cigarettes used in this adaptation session will be the same as those used in the test sessions. Although the familiarization with this technique will occur during the adaptation session, participants will be reminded of this inhalation technique during each test session just prior to e-cigarette use. Participants will be informed that the schedule of flavor delivery prior to each IV nicotine infusion will consist of 6 inhalations at a rate of one inhalation every 15 seconds.

b) Test Sessions: Experimental sessions will start around 8:30 AM. As summarized in Table 2, blood samples for baseline biochemical measures, including plasma cotinine, nicotine, and for women, estradiol and progesterone levels, will be obtained just prior to the collection of baseline subjective ratings and cognitive testing. Subjects will then start inhaling from the e-cigarette with the assigned flavor condition (menthol, green apple or menthol plus green apple), one inhalation every 15 seconds for 90 second as practiced in the adaptation session (6 inhalations total (Vansickel *et al*, 2012). The e-liquids used in the e-cigarette will be prepared just prior to each test session by the VA Research

Pharmacy. Each e-cigarette tank will contain 2 mL of the e-liquid and the study personnel will load the tank into the e-cigarette. Just after the last of the 6 flavor inhalations, participants will then receive the first delivery of the assigned nicotine infusion (saline, 0.25 mg/70 kg or 0.5 mg/70 kg nicotine). Each infusion will be 60 minutes apart to provide sufficient time for the physiological and subjective nicotine effects to return to baseline. Following each infusion, physiological, subjective, and cognitive outcome measures will be obtained.

c) Medical Monitoring: For all nicotine test sessions, subjects will be attached to a blood pressure and heart rate/rhythm device (3-lead EKG) that will be monitored by the study physician. An IV catheter will be in place throughout the session. Subjects will be administered nicotine only if the systolic blood pressure is <150 mmHg and heart rate is <100 beats/minute. Subjects will be terminated from the study if the blood pressure at any time is >170/110 mm Hg, the heart rate is >120 beats/min, or if they develop signs and symptoms compatible with nicotine toxicity. Subjects will remain in the laboratory for two hours after the last nicotine administration. These procedures have been developed as part of our IND application for IV nicotine.

Table 2. Schedule of Events: Experimental Session*

Time	Measures and Events
8:00 AM	(Building 36) CO level, urine sample, BAL, and light standard breakfast
9:00 AM	(Biostudies Unit) blood sample, HR/BP, EKG, M-NWSC, BQSU, SAFTEE, Cognitive Testing
10:00 AM	Flavor (menthol, green apple or green apple + menthol) DEQ + IV Saline or Nicotine (0.25 or 0.5mg/70 kg) blood draw
10:01	HR/BP, DEQ
10:02	HR/BP
10:03	HR/BP, DEQ,
10:05	HR/BP, DEQ
10:08	HR/BP, DEQ blood draw
10:10	HR/BP, DEQ
10:15	HR/BP, DEQ, Cognitive Testing
10:30	HR/BP, DEQ blood draw
10:45	HR/BP, DEQ
10:55	HR/BP, DEQ, BQSU
11:00	Flavor (menthol, green apple or green apple + menthol) DEQ + IV Saline or Nicotine (0.25 or 0.5 mg/70 kg), blood draw
12:00	Flavor (menthol, green apple or green apple + menthol) DEQ + IV Saline or Nicotine (0.25 or 0.5 mg/70 kg), blood draw
13:00	HR/BP, EKG, M-NWSC, BQSU, SAFTEE, blood draw
14:00	Discharge

*The same measures will be obtained following saline and each nicotine administration. For brevity, only the measures after saline are shown. Abbreviations: CO: Alveolar carbon monoxide; HR/BP: Heart rate/Blood pressure; EKG: Electrocardiogram; M-NWSC: Minnesota Nicotine Withdrawal Symptom Checklist; BQSU: Brief Questionnaire of Smoking Urges; DEQ: Drug Effects Questionnaire; SAFTEE: Systematic Assessment for Treatment Emergent Events.

12. Data Analysis Methods

Data analyses: The primary analyses will be intent-to-treat and will include all available data on subjects who complete at least one test day. Mixed-effects models will be used to test the study hypotheses. These models allow for different numbers of observations per subject, use all available data on each subject, and are unaffected by randomly missing data. They also provide flexibility in modeling the correlation structure of the data. All data will be checked for

normality and transformations will be applied as necessary. Significance level of 0.05 will be used for the main hypotheses and Bonferroni correction will be applied for post-hoc tests.

Specific Aim #1: To examine the acute reinforcing effects of menthol, a fruit flavor (green apple) or a fruit flavor plus menthol, alone or in combination with nicotine in smokers. Menthol and fruit flavor will be administered by inhalation via electronic cigarettes (e-cigarettes) and nicotine will be administered intravenously. The reinforcing drug effects will be measured with the drug effects questionnaire (DEQ).

Hypothesis:

Hypothesis #1A: Green apple plus menthol will be more reinforcing than menthol alone or green apple alone. Hypothesis #1B: Green apple plus menthol will be more effective than menthol alone or green apple alone in enhancing the reinforcing effects of nicotine.

The main outcome measures will be the "good effects" and "drug liking" items of the DEQ. Mixed effects models will include the within-subject factors nicotine dose (0.25, 0.5 mg nicotine or placebo), flavor (menthol, green apple or menthol + green apple) and time of measurement (+1, +3,+5,+10,+15,+30,+45,+55 minutes) and possible interactions among these factors. We will also consider order effects for nicotine dose (1, 2 or 3) and session day (1, 2 or 3) and the effects of potential covariates (race, sex, baseline plasma NMR, FTND scores). These effects will be included in the final model if significant. Estradiol and progesterone levels will also be considered as potential covariates in a secondary analysis of women only. We will consider several different correlation structures for the repeated measures within individuals, and will select the best fitting one based on Schwartz Bayesian information criterion. SAS PROC MIXED will be used for the mixed-model analyses.

Rationale for sample size: The study will have 80% power to detect large effect sizes (f=0.4) for the interactions of interest in repeated measures analysis for 30 subjects at 0.05 significance level. Our preliminary data for both outcome measures show larger magnitude effects for the nicotine effects (all f>0.5) and flavor effects are expected to be similarly strong (Sofuoglu et al. 2009a). Applying Bonferroni correction and using an alpha level of 0.01 we have 80% power to detect post-hoc comparisons of medium to large effect size d'=0.7 for flavor and nicotine effects. Our previous study shows larger effects of nicotine d'>1.0. To account for up to 20% dropout we will recruit 36 subjects for a target of 30 completers.

13. Risks and benefits

Potential risks:

There are potential risks, discomforts and inconveniences associated with participation in this study. These may be due to nicotine or menthol administration, blood drawing, other study procedures and potential loss of confidentiality.

1) Common side effects of nicotine include nausea, vomiting, heartburn, and elevated heart rate and blood pressure. Toxic doses of nicotine may cause abdominal pain, hyper salivation, diarrhea, dizziness, confusion, hearing and vision problems, syncope, seizures, hypotension, irregular pulse, and death. However, these toxic effects occur at doses 40 to 50 times that which will be used in our study. Other potential risks from this study include administering a drug that has addictive potential.

2) Menthol administration produces typical sensory effects in the mouth and throat. The doses of menthol that are found in cough drops, ranging 1 to 10 mg are regarded to be safe (Sweetman, 2011). In spite of ubiquitous use of menthol in a wide range of products, only few cases of menthol poisoning has been described in the literature following very high doses of menthol ingestion, 200 mg or more. Menthol poisoning reported to cause ataxia, confusion, coma, nausea, and vomiting. However, these toxic effects occur at doses 20 to 30 times that which will be used in our study.

3) Inhalation of two of the e-liquid flavors will contain a fruit flavor. All flavorants and sweeteners contained in the fruit flavor are classified by the FDA as Generally Recognized As Safe (GRAS). Fruit flavors are commonly used chemicals used in a wide array of food and cosmetic products. Although it is rare, allergic reactions to fruit flavors can occur so if a subject has a bad reaction or are allergic to products with the relevant fruit flavor or aroma, they will be excluded from study participation for their safety. However, the administrations will be closely monitored for any signs that this unique combination of IV nicotine and inhaled flavorants results in unexpected findings.

4) Propylene glycol and vegetable glycerin are categorized by the FDA as compounds that are Generally Recognized As Safe (GRAS). They are both commonly used ingredients in food products, pharmaceuticals and in personal care products. They are also now used as the base liquid ingredient for e-cigarettes. Although e-cigarettes are reported to be helpful for smoking cessation, the FDA has not approved e-cigarettes for this use, and the long-term consequences of e-cigarette use have not been assessed.

5) Blood Drawing: Subjects will have approximately 260 mL of blood drawn as a result of their participation in the study. Blood drawing can cause some pain and may result in bruising.

6) Study procedures: On the test days, subjects will not be able to smoke for more than 10 hours. During this cigarette abstinence period, subjects may experience symptoms of nicotine withdrawal such as craving for cigarettes, anxiety, restlessness, irritability, difficulty concentrating, loss of energy, and excessive hunger. In addition, smokers will be exposed to e-cigarettes and e-cigarette mixtures.

7) Loss of confidentiality: It is possible that participation in the study may make others, including friends or family members, be aware of the participant's tobacco use status. The potential participants will be told that if they do not feel comfortable with this then they should not participate in the project. Participants will also be told that if they report any information to us about abuse or homicidal/suicidal behavior we will be required to report this information to the appropriate authorities. There is also a potential risk of loss of confidentiality due to data sharing.

14. Protection of Subjects:

Before initiating any research activity, each subject must give informed consent that will detail the risks of study participation. They will also be advised of the data sharing plans for the study. Eligibility will be determined by the medical and psychiatric history, drug use history, the physical examination, and the laboratory studies done prior to beginning this research protocol. This study will enroll young adults between the ages of 18 to 30 because of the high prevalence of use of mentholated tobacco products in this age group (Rock *et al*,

2010). To minimize the risks associated with administering a drug that has addictive potential, we will only enroll those individuals with a consistent history of cigarette smoking as verified by a careful history, questionnaires, and urinary cotinine levels. Additionally, we will not enroll treatment-seeking smokers.

As mentioned previously, the most common expected adverse events related to IV nicotine administration are nausea, vomiting, heartburn, and elevated heart rate and blood pressure. To prevent nausea, subjects will be asked not to eat before the sessions. In addition, subjects will sit in a comfortable reclining chair during the test sessions that minimizes heartburn. Subjects will also be carefully screened for the presence of high blood pressure or other cardiovascular disorders to minimize cardiovascular adverse events.

During the test sessions, a physician will closely monitor all subjects. Subjects will be attached to a cardiac monitor as well as a blood pressure and heart rate monitoring device. An IV catheter will be in place throughout the session. Stopping rules are in place as described above (Study Procedures - Test Session).
Over the last 6 years, we have administered nicotine intravenously to more than 400 smokers and have not encountered any adverse events attributed to nicotine administration. The most common adverse events, or reasons for discharges, in these studies include difficulty finding venous access, high blood pressure before nicotine administration, the use of drugs of abuse or alcohol during study participation, and adverse events attributed to other study medications. Similar to our experience, in a genetic study, Gary Swan et al. administered IV nicotine to 278 smokers and non-smokers via slow infusion (Cancer Epidemiol Biomarkers Prev 2007;16(6):1057–64). This procedure was also well tolerated in both smokers and non-smokers, further supporting the safety and tolerability of IV nicotine administration.

The close monitoring of subjects during the sessions will also help to detect any adverse events from menthol or e-cigarette administration.

Confidentiality will be protected by having records identified by code number only with the master list including names kept in a sealed envelope in a locked file in the Principal Investigator's office and by the pharmacy. Subjects will be given telephone numbers to call in case of emergency, 24 hours a day. The potential risk of loss of confidentiality due to data sharing will be minimized by creating de-identified data sets that exclude direct and indirect identifiers. In addition, researchers requesting data will be required to enter into a data sharing agreement.

In order to participate in a study, each subject must give informed consent. All potential risks will be described in detail to the subjects in the consent form. The personnel in the laboratory have been certified in either Advance Cardiac Life Support (ACLS) or Basic Life Support. If a problem arises, the subject will be treated immediately.

Confidentiality in this study is of the utmost importance to us. All information obtained will be stored in coded form. The names of the subjects will be used in hospital records.

The risk associated with participating in this study is moderate, because the nicotine administered may be associated with mild side effects. Serious side effects associated with this treatment are not expected. This project will be monitored by the Center’s Data and Safety Monitoring Board (DSMB) because the study involves double-blind treatment of smokers with nicotine. This board is composed of persons not otherwise affiliated with the

clinical study who are experienced in various aspects of the conduct of clinical trials for the treatment of addictive disorders. The Yale TCORS Independent Data Safety Monitoring Board includes experts in the field of tobacco use behaviors and challenge studies (Chair: Dr. Tony George, FRCPC, Professor and Co-Director, Division of Brain and Therapeutics, Dept. of Psychiatry, U of Toronto; Dr. Thomas Brandon, Professor and Chair, Department of Health Outcomes & Behavior, H. Lee Moffitt Cancer Center & Research Institute) and in statistics (Dr. Hanga Galfalvy, Assistant Professor of Neurobiology, Columbia University). The members of the DSMB, and all study Investigators, will complete Conflict of Interest forms created by Yale's IRB in accordance with NIH guidelines.

We will report recruitment, follow-up, and adverse events to this panel on a bi-annual basis with serious adverse events reported within 48 hours. Prior to study initiation, critical parameters for collection of side effects and for study discontinuation will be recommended to the DSMB who may use these or other measures to monitor safety of the ongoing trial. The DSMB will be available to convene outside of scheduled meetings, if necessary, due to concerns regarding a particular subject or due to any troublesome developments in subjects' experiences during the study. The DSMB will make appropriate recommendations for changes in the study protocol, if needed.

This monitoring will be consistent with NIH policy regarding the protection of human subjects in research, and FDA guidance on statistical practices for clinical trials (ICH E9) and good clinical practices (ICH E6). In general, the data to be reviewed will include screening data, baseline data, efficacy data, and safety data.

The Principal Investigator will conduct a review of all adverse events and determine the attribution and grade of severity of the adverse event by using the following scales:

Attribution of Risk Categories:

Definite: Adverse event(s) will clearly be related to investigational agent(s) or other intervention

Probable: Adverse event(s) will likely be related to investigational agent(s)

Possible: Adverse event(s) may be related to investigational agent(s)

Unlikely: Adverse event(s) will doubtfully be related to investigational agent(s)

Unrelated: Adverse event(s) will clearly not be related to the investigational agents(s)

Grades of Risk:

0: No adverse event or within normal limits

1: Mild adverse event

2: Moderate adverse event

3: Severe adverse event resulting in hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect

4: Life-threatening or disabling adverse event

5: Fatal adverse event

Serious adverse events (SAEs) include any untoward medical occurrence that at any dose results in death or the immediate risk of death, hospitalization or the prolonging of an

existing hospitalization, persistent or significant disability/incapacity or a congenital anomaly/birth defect. Subjects will be terminated from participation if the investigator feels that subjects' health or well-being may be threatened by continuation in the study. Serious unanticipated and anticipated adverse events will be reported within 48 hours to the VA Hospital, Yale IRB, the DSMB, and NIH. We will directly report to the FDA, whenever their magnitude or frequency exceeds expectations.

15. Informed Consent: Recruitment will be by word-of-mouth, referrals from area programs and by advertisement. A research staff member will interview individuals that are interested in participating in the study over the phone. If subjects pass the initial screening for the study, they will then come into the clinic for a full screening evaluation. Upon arrival, research assistant will read the detailed consent form and will ask questions to make sure that the subjects understand the procedure, and their rights and informed consent will be obtained.

16. Confidentiality: Only authorized research personnel will have access to the information gathered in this study. Patient names or other identifiers such as social security number, initials, and birthdate will not be used to identify paper or electronic records. Only a code number will identify the individual research records. The code number will not be based on any information that could be used to identify the subjects (for example, social security number, initials, birthdate, etc.). The master list linking names to code numbers will be kept separately from the research data and will be stored in an electronic file behind the VA firewall. All research information obtained will be kept in locked cabinets located on VA property or in electronic files behind the VA firewall. The names of the subjects will be used in VA electronic records, CPRS.

17. Location of Study: This study will be conducted in Ward G9W (the Biostudies Unit) located in Building 1 at the West Haven VA Medical Center.

18. Payment: Subjects will be paid $30.00 for attending the screening visit and $30 for the Adaptation Session. Subjects will be paid $150 for each of the three Test Sessions. In addition, subjects will be paid $20 to cover transportation costs for the Adaptation Session and for each Test Session. Completing all components of the study will result in a maximum total payment of $590. In addition, subjects may also earn $20 for referring people they know who also smoke cigarettes and are eligible for study participation. A contingency payment of $20 will be given for transportation if a subject needs to be re-contacted. We will not share any information with the subject who makes the referral about the ultimate enrollment status or study outcome of the referred individuals.

Payment Schedule

Visits	Amount Paid	Total
Screening Visit	$30.00	$30.00
Adaptation Visit	$30.00	$30.00
Travel for Adaptation, and Test days 1,2, and 3	$20.00 each visit	$80.00
Test days 1,2 and 3	$150.00 each test day	$450.00
	Max Total Payment	$590.00
Referral	$20.00	$20.00

19. Funding Source: Provided by an NIH Center Grant.

20. Duration: The entire study will take approximately one and a half years to complete.

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