The Ohio State University

The Effects of a Standardized Research E-Cigarette on the Human Lung:
A Clinical Trial with Bronchoscopic Biomarkers

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Revision History:

Revision Summary	IRB Approval Date
Original submission	**03/05/18**
Removed references to “FDA approval”; Updated study schema to clarify timing of study procedures; Revised eligibility criteria (age range extended to 45; Exclusions: regular consumption of roll your own cigarettes; steroid use in the past 30 days; allergies to propylene glycol, glycerin, or flavors; added THC strip tests for marijuana exclusion; adverse reaction to previous e-cig use; specified a previous three month period for pregnancy or breastfeeding); Revised advertisement language to reflect age range; Added language to clarify an additional two tubes of blood will be collected for the nicotine boost; Added description of product labeling by manufacturer and product dispensing information; Added the corresponding week to the existing day associated with study procedures (e.g., Day 1/Week1); Added a window in which visits and calls can occur without being considered a protocol deviation; Updated measures table; Added a planned analyses table; Removed language regarding the blood draw being done by IV; Added proteomic and CyTOF analysis; DSMP reflects appropriate changes listed above and removed Anita Kinney.	**07/02/18**
Extended the intervention period from 4 to 8 weeks and updated measures to harmonize study with other NIDA grantees; Revised timing of intervention activities (e.g., randomization) to better align with duration on product; Revised subject incentive plan to include retention and compliance bonus; Added varenicline use to foster compliance in the nicotine-free SREC group and indicated that a history of hypersensitivity to varenicline will be considered as an exclusion criteria; Clarified the quantity of the study products and medication to be dispensed at each visit; Listed risks related to varenicline use, monitoring by the study; Added CO measure; Added mtDNA description; Moved CyTOF and proteomics descriptions to be consistent with existing format of the protocol; Updated study figure and clarified patients can contact staff for additional cessation counseling support as needed; Clarified the contact efforts to schedule or reschedule subjects for planned visit or call; Removed clinical consent process at the recommendation of clinical staff and Dr. Wewers as it was determined to be redundant and will now better align with other bronchoscopy studies being conducted at the CRC; Clarified THC will be measured at each visit; Added using an instructional video showing how to use SREC device; Added monitoring in-person SREC use through a webcam, to ensure adequate device use during inhalation assessment as needed; Added the possibility of providing cell phones to subjects who indicate difficulty maintaining a reliable source of communication after enrolling in the study to facilitate communication, data collection, and improve retention, Removed Dr. Jackson from the study key personnel; and Updated sample size and power.	**08/24/18**
Eligibility criteria updated to allow other tobacco use up to 10 times in three months but no use within 2 weeks and the marijuana exclusion was revised to limit the exclusion to daily or most days use and removing the positive test component since testing will remain but only to be used in analyses; Added Information Warehouse and Social Media recruitment; Clarified language regarding the temperature of saline used for nasal lavage (room temperature); Added youtube link for	**12/06/18**

instructional video for SREC users; Clarified that subjects who were not able to reduce to 5 cpd or less will remain in the study; Added participants will be asked to return all unused product and the devices at the end of the study; Clarified language for varenicline dispensing to allow for a 4 week cards/blister packs to be provided and updated NDC; Clarified NRT dosing, maximum amounts, and option for combination therapy; Added option for PI to remove subjects who are non-compliant; Updated measures table; Clarified nicotine boost procedure to instruct use of device prior to visit; Clarified language regarding use of spirometer during inhalation assessment; Updated saliva collection to only ORNT, BR1, BR2; Added THC to lab procedures; Updated total amount of time required for subjects; Clarified how data are stored and protected by removing redundant information regarding database and adding information on the information warehouse and social media data; Updated risks to include poisoning from e-liquid, fires or burns from device and long-term e-cig use, and explaining NRT is safer than smoking; Added or updated forms corresponding to changes above.	
Added Min-Ae Song as a co-investigator; Updated design to include new arms (e.g., Juul and IQOS) and removed dual use arm (6 arms: continued smoking, nicotine SREC, nicotine-free SREC, Juul, IQOS, NRT) - this approach adds new devices commercially available for comparison, and the design allows for a rolling admission of products and increased contemporary controls in the event a device is not available at the time of enrollment; Increased sample size to 40 per group, n=240 to allow for a rolling admission of new conditions and increased controls; Clarified procedures to protect privacy; Clarified that subjects will be asked to refrain from using marijuana and all other tobacco products while active in the study (however, use is not an exclusion); Removed all references to marijuana exclusion; Removed THC strip tests for marijuana exclusion (laboratory analyses will be conducted, but not used for exclusionary purposes – allows the subjects recruited to better reflect the population at large); Clarified the duration of the regular use of inhalant medications (3 months); Added a history of bronchospasm to the exclusion criteria for participant safety; Added descriptions of the Juul and IQOS products along with anticipated distribution; Removed narrative list of measures as it was redundant with existing Table 1; Clarified the list of tobacco use biomarkers planned; Revised the number of bronchoscopies to “over 100” as the number continues to increase; Clarified description of analyses for bronchoalveolar lavage cells and saliva for RNA microbiome; NicAlert test (urine/saliva) - removed from eligibility criteria; Updated advertisement language to potentially include statements that clarify participants will not be excluded for using marijuana - to better identify potential subjects and reflect the population as a whole; Noted that ads will be linked to a landing page (http://go.osu.edu/srec-study) and short survey; Noted that study staff will be blinded regarding study groups, and medical personnel will be informed if s/he deems it pertinent – further protect confidentiality; Added current FDA and CDC recommendations regarding EVALI to inform subjects of the current scientific knowledge; My Chart recruitment to increase sample selection; Added 24 hour urine collection and urine osmolality to further assess biomarkers; Added that upon prior arrangement, subjects without a ride home from the procedure may stay at the CRC under supervision by CRC staff for 24 hours, and this extended stay will be paid for by the study to accommodate patient needs and increase the pool of subjects who may be able to participate; Updated list of measures: Alcohol and drug abuse history, supplement use, general diet and physical activity, 24-hour urine collection questionnaire, lung randomization and BAL collection information, and SREC, Juul and IQOS weight log; Clarified that IHIS may be used to facilitate lab processing with pathology, but no other access to medical records will be required for this study due to some study samples being examined by OSU	**05/14/2020**

pathology and the recent change in IHIS reporting (Beaker system). Updated e-cig/vaping risks; Risk section was updated to generalize previous experiences and emphasize that there have been no SAEs; Statistical methods were updated to reflect the study design; Updated handouts and links for device materials; Edited study documents to reflect changes described above: consents, eligibility checklists, brief medical history, tobacco use history (replaced), daily use summary and sample collection form.	
Limited arms to 4 (control, SREC, placebo SREC, NRT); Kept the language about the option to add in other products as needed; decreased the sample size in each group (new n=32 x 4); Clarified that subject's medications, herbs, supplements and vitamin will be reviewed by Dr. Shields and Wewers, and that subjects will be informed if there are any of their regular medicines, herbs, supplements or vitamins they should or should not take before their bronchoscopy; Updated study schema to clarify timing of study procedures; Updated ITP statement; Viral exposure precautions with the caveat that we would follow institutional procedures that may evolve over time; Added that all Subjects will be given a monitor to measure their breath CO level at home and will be asked to download iCO app on their electronic device (iOS or Android) to report their breath test daily; Revised eligibility and exclusion criteria to include and clarify the requirements of COVID 19 test and breath CO daily report; Added electronic consent (will still be in-person); Updated the locations of all in-person visits and bronchoscopy; Moved the nicotine boost procedure to be done at Day 57/week 8 visit, instead of BR2 visit; Removed the requirement of patients to sign the medical center clinical procedure consent form before bronchoscopy; Updated device and product information; Removed specific information about Juul and IQOS; Revised subject incentive plan and clarified the compliance bonus; Updated Risks and Protections; Updated Statistical methods to reflect the study design. Clarified that the bronchoscopy procedure and potential need for COVID-19 precautions may require accessing the participants electronic medical records through IHIS, and results from bronchoscopy and COVID-19 test may be included in the participant's medical record, but only authorized study personnel will have access to the data; Edited study documents to reflect changes described above: consents, eligibility checklists, evaluation questionnaire, weight, height and recent product use, product accountability log, e-cig cartridge/pods weight log, final subject status, advertisements, phone guide, and subject's handouts; DSMP reflects appropriate changes listed above; Reformatted some of the demographics questions for consistency.	**04/12/2021**
Updated ITP statement; Limited FeNO measurement and urine sample collection to the orientation and bronchoscopy visits only; Added Covid-19 Screener and Covid-19 follow up to the table of measures; Deleted general diet and physical activity from the table of measures as it is included in the pre bronchoscopy questionnaire; Updated study schema and table 1, 2 and 3 to clarify timing of study procedures; Renamed SREC Evaluation Scale and SREC Cartridge/ Pod Weight Log to Evaluation Questionnaire Electronic Vaping Devices and E-cig Cartridge/ Pod Weight Log, respectively, to match the corresponding data collection forms; Revised the language for compliance bonus; Add Oil-Red-O to the lab analysis table under BAL where missed; Added a language to clarify data sharing and publication; Edited study documents to reflect changes described above or for	**11/05/21**

consistency: consent, Covid-19 surveys, health changes questionnaire, Daily Use Summary, Evaluation Questionnaire Electronic Vaping Devices, E-cig Cartridge/ Pod Weight Log, inhalation assessment, instructions script, advertisements, added an electronic consent email template, and added Covid-19 test appointment notification email/letter template. Updated study key personnel.	
1. **Study Summary**: Clarified saliva collection *-Inadvertently not included in list of samples for repository* 2. **Eligibility**: Age increased to 52; Number of cigarettes smoked per day decreased to ≥5 *-Increase the participant pool and to make results more generalizable to the general population of smokers, as a response to reviewers' comments* Clarified that smokers who smoke cigarettes rolled by hand will be excluded *-Clarified phrasing to distinguish from roll your own cigarettes created using a machine* 3. **Recruitment**: Added Respondent Driven Sampling (RDS) *-Smokers are likely to know other smokers who are potentially interested in participating. This sampling method is a type of peer-driven chain-referral sampling (Heckathorn 1997, 2002), that has been used in other studies at OSU and has been successful in identifying hard to reach populations.* Revised ad language compensation *-Reflect changes in compensation* Clarified the EMR number will be included in the IW request *-HBOC requires this field to track the patient's vital status* 4. **Consent Process**: Added the option to review the study details and ICF remotely prior to the orientation visit *-Reduce participant burden* *5.* **Compensation**: Increased payments and option to utilize a transportation service provided at no cost *-Reflect additional burden/costs associated with time and travel* 6. **Study Procedures**: Modified FeNO measurement and urine sample collection to the bronchoscopy visits only *-Reduce participant burden* Moved inhalation assessment for all SREC arms and nicotine boost for nicotine-SREC arm to the BR2 visit only *-Reduce participant burden* Revised the description of the nicotine boost *-Updated procedure and clarified process* Changed "will" to "may" when describing the duration of the bronchoscopy procedure *-The procedure depends on multiple variables and is often very short*	**09/19/2022**

URL provided for the approved instructional video *-Previous place holder* Clarified combination therapy language *-Better describe preferred and alternative options* Added option to complete questionnaires online and measure CO within 24 hours before scheduled visit *-Reduce participant burden* 7. **Data Collection Timeline**: Updated timing *-Reflect additional time necessary for some subjects who may be slower to complete study/procedure tasks and for precautions related to COVID-19/viral illnesses* 8. **Protection Against Risks**: Added RDS *-Address privacy* Replaced Dr. Wewers with Dr. Tsai *-Updated study personnel* 9. **Data Analysis Plan**: Added monitoring of RDS *-Identify/address bias if appropriate* Sample size and power updated *-Reflect attrition* 10. **Study Overview**: Updated sample collection, nicotine boost, inhalation, and FeNO *-Reflect changes described above* 11. **Tables 1, 2 and 3**: Updated timing of procedures and group-specific questionnaires *-Reflect changes described above and reduce participant burden* Corrected typos throughout the document 12. **Study Documents**: Edited ICF, measures, advertisements, phone guide, IW-data sheet and provider script, SAP and DSMP *-Reflect changes described above* 13. **Additional Files**: SREC Screener; Study Information PowerPoint; RDS Flyer/Coupon; Parking and Walking Direction to CRC, and Excuse letter *-Facilitate participation and reduce participant burden* **14. Key Personnel updated**	

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1. Introduction / Overview

The use of electronic cigarettes (e-cigs) is increasing rapidly. Yet, little is known about the potential lung toxicity for inhaling e-cig aerosols relative to smoking. The Food and Drug Administration (FDA) has deemed regulatory authority over e-cigs and needs data to determine how to regulate their designs and constituents. It is plausible that e-cig aerosol constituents (carriers, flavor, and byproducts of these) induce an inflammatory response in the lung, but there is no direct evidence for this, and what the magnitude of the effect is, if any. While there may be numerous ongoing laboratory *in vitro* and *in vivo* studies to assess e-cig exposure and toxicity, these utilize indirect methods for assessing lung toxicity. There also are ongoing human studies that focus on the effect of e-cig use on smoking-related biomarkers of exposure, but these usually assess biofluids outside the lung (e.g., blood and urine). It is unknown how any of these studies may relate to lung toxicity, i.e., the target organ, and thus there is a critical research gap. To assess human lung toxicity from e-cig use, we propose to conduct a clinical trial of 128 smokers to assess inflammatory responses in the lung via serial bronchoscopy. To do this, we will exploit an extraordinary opportunity for study e-cig toxicity by employing a new standardized research e-cig (SREC), a recently developed e-cig by the National Institute on Drug Abuse (NIDA) that will be submitted to the FDA for use as an investigational tobacco product. (This protocol is a NIDA-funded study, and OSU was only one of 3 groups nationally selected to investigate this product.) The study will also utilize a design that will allow for continual recruitment of control subjects with the potential to add new arms/conditions as novel products enter the market. This protocol may be amended as new products become available. This design will facilitate timely and cost-efficient investigation. In the current iteration, subjects will be randomized to continued cigarette smoking (control), one of two e-cig use conditions (complete substitution with the nicotine-SREC or placebo-SREC), or complete substitution with nicotine replacement therapy (NRT). Each group will be 32 subjects and the trial will be 10 weeks (a 2 week run-in period, followed by 8 weeks of the experimental condition). Primary outcomes will be lung biomarkers (cell counts and inflammatory cell subtypes, altered macrophage phenotypes such as lipid-laden macrophages, inflammatory cytokines, exhaled nitric oxide, mRNA and microRNA gene expression, DNA methylation, acrolein DNA adducts, proteomics, and mitochondrial DNA mutations and copy number variation, lipidomics, gene methylation, microbiomes, nicotine and carcinogen metabolites, CyTOF, proteomics, propylene glycol and glycerin, and untargeted metabolomics). Pulmonary function tests also will be assessed. Several biochemical measures to assess compliance will be employed (e.g., exhaled Carbon monoxide (CO) level, urinary tobacco alkaloids, nicotelline, nicotine boost, propylene glycol and glycerin). A biorepository of urine, blood and nasal samples will be created for future studies to compare non-invasive biomarkers with lung biomarkers. The infrastructure for this trial that investigates the pulmonary effects of e-cig use among non-smokers is already established under an FDA- and OSU IRB approved protocol (OSU-2015C0088; Clincaltrials.gov NCT02596685), which has completed more than 100 bronchoscopies. This study is significant because it will focus on lung inflammation, a plausible effect on the lungs of e-cig use, and the impact for switching to e-cigs, versus complete cessation with NRT. Such data are needed as soon as possible to assist the FDA in the regulation of e-cigs, including nicotine content. It also will provide critical data to NIDA about the use of their product and smoking behavior for other studies they intend to fund in the future. The study is innovative by using a clinical trial design using serial bronchoscopy, incorporating inflammation biomarkers with metabolomic and gene expression data, and uses an integrative, complementary and comprehensive approach through multiple 'omics assays. It also is innovative because it will provide unique data for the effects of nicotine on the human lung for inflammation and other pathways. This study has considerable public health impact because it will provide experimental evidence in humans of target organ effects.

Note: This study will use the NIDA SREC, which is currently modeled after NJOY's commercial e-cig product, the Ace. This device will be used in research studies via an Investigational Tobacco Product (ITP) designation.

On April 4, 2018, the FDA issued a Letter with No Concerns regarding the ITP application that was submitted concurrently with the original IRB application. The study was initiated, but temporarily suspended while modifications to the SREC product were made. As such, a new ITP application was submitted at the time of the January 2021 IRB amendment and a second Letter with No Concerns was issued on March 26, 2021.

2. Specific Aims / Objectives

Electronic cigarettes (e-cigs) deliver nicotine, flavorings, and carriers through heated aerosols. The use of e-cigs generally, and as an occasional and regular use alternative to smoking, is growing at an extraordinary rate. The FDA has recently deemed the authority to regulate e-cigs, but regulatory decisions require safety data. It is plausible that the e-cig use will induce lung toxicity by promoting inflammation, but data for this is a critical research gap. Studies of healthy smokers undergoing bronchoscopy demonstrate an effect on promoting inflammation, which is an important component for the pathogenesis of chronic obstructive pulmonary disease and lung cancer. It is plausible that e-cigs affect similar mechanisms, albeit at a lower amount. E-cig human biomarker studies (cross-sectional studies and clinical trials) demonstrate clear decreases in cigarette smoke exposure toxicants with complete switching. However, these studies assess blood and urine biomarkers, where it is unknown if the biomarkers are surrogates for lung biomarkers and inflammation.

In this application, we can take advantage of a new opportunity using the newly released National Institute on Drug Abuse (NIDA) standardized research e-cig (SREC), because this is a product that will likely not change over time subject to marketing and manufacturing influences, and allows for comparisons across studies. It also is available with and without nicotine. While there are numerous experimental laboratory studies underway to infer human lung toxicity (e.g., chemical, cell culture and animal studies about e-cig aerosol constituents), regulatory decisions will best be made with human data for actual e-cig use. It is well-established that cigarette smoke increases pulmonary inflammation contributing to lung disease and cancer. The availability for the SREC with and without nicotine provides a unique opportunity to assess the contribution of nicotine, a highly bioactive molecule, to lung toxicity. Nicotine is both pro- and anti-inflammatory, but more data weighs in for an overall anti-inflammatory effect and so that will be studied herein and hypothesized to attenuate the pro-inflammatory effects of e-cig aerosols.

We propose to assess pulmonary effects of e-cig use in a randomized clinical trial of 128 smokers, some of whom will be provided e-cigs, such as the SREC, for 10 weeks (2 weeks run-in period and 8 weeks of the experimental condition) assessing biomarkers obtained by bronchoscopy (bronchoalveolar lavage [BAL] and lung brushings). Participants will be randomized to one of four groups of subjects (n=32 for each group): a control group who will continue to smoke their usual brand, a group who will switch completely to the SREC with nicotine, a group who will switch completely to the SREC placebo and a group who will stop smoking using nicotine replacement therapy (NRT). Using serial bronchoscopy, we will assess pulmonary inflammatory biomarkers and 'omics to provide direct evidence of the effects of e-cigs, with and without nicotine, compared to continued smoking and complete cessation. Using bronchoscopies at baseline and after 8 weeks of the experimental condition (after a 2 week run-in period), the primary outcomes will be a change in inflammatory cell counts and inflammatory cell subtypes, altered macrophage phenotypes such as lipid-laden macrophages, inflammatory cytokines, fractional exhaled nitric oxide (FeNO), mRNA and microRNA (miRNA) gene expression, gene methylation, DNA methylation, acrolein DNA adducts, proteomics, and mitochondrial DNA mutations and copy number variation, lipidomics, microbiomes, nicotine and carcinogen metabolites, CyTOF, proteomics, propylene glycol and glycerin, and untargeted metabolomics. Pulmonary function tests (PFTs) also will be conducted.

Compliance and comparisons with interview data will be assessed by measuring chemical exposures, such as but not limited to THC, exhaled CO, cotinine, tobacco biomarkers, propylene glycol and glycerin. We will also utilize a repository of data and biosamples such as urine, blood, nasal epithelia and nasal lavage for future studies to assess non-invasive biomarkers as possible surrogate biomarkers for lung toxicity. The infrastructure and feasibility for this study has been established through a pilot study funded by our NIH P50 Tobacco Center of Regulatory Science (TCORS) that received FDA approval as a TCORS pilot project, is registered under Clincaltrials.gov NCT02596685, has undergone competitive peer-review, and has been approved by our university IRB under OSU-2015C0088. The primary hypothesis of the pilot project was that e-cig constituents induce an inflammatory response in the lungs of never-smokers, which is less than or different from that of cigarette smoking. Our current preliminary data from a cross-sectional study of smokers, non-smokers and e-cig

users indicate that e-cig users have less inflammation than smokers, and more than non-smokers. The funding for this current SREC-use protocol for smokers has been obtained by NIH peer-review (U01 DA045530).

The hypothesis for this study is that human lung toxicity will decrease for smokers substituting e-cigs for smoking, but the effect will be less for the e-cig devices compared to quitting with NRT. The effects will be lessened for e-cigs without nicotine because of the anti-inflammatory effects of nicotine. This study is highly significant because it will provide direct evidence for the effects of e-cigs on inflammatory pathways in the human lung among smokers, and identify the magnitude of reduction (if any), facilitating FDA decision making for product designs. We will have scientific rigor by using a randomized group assignment and laboratory quality control measures. The data will be useful for future studies using the SREC and commercially available e-cigs differing in design, voltage and constituents, and studies of longer duration. This study is innovative because it will provide experimental evidence in humans for target organ effects, has a focus on smokers, and incorporates inflammation with untargeted metabolomic and gene expression data allowing for a complementary and comprehensive approach for data integration (e.g., does the combination of several 'omics types better distinguish treatment effects). Notably, this application responds to PAR-17-156: "Evaluating the NIDA Standardized Research E-Cigarette in Risk Reduction and Related Studies", and the NIDA research agenda by directly addressing the PAR's goals for assessing the SREC and pulmonary research. The Specific Aim for this two year project is:

1. To assess inflammatory changes over 10 weeks (8 weeks experimental condition) for lung and urine biomarkers in smokers in a clinical trial where subjects are randomized to continued use (n=32), complete switching to the nicotine SREC (n=32), complete switching to placebo SREC (n=32), or complete switching to NRT (n=32).

3. Background and Rationale

Electronic cigarettes (e-cigs) are a wide variety of products that result in aerosolizing (vaporizing) nicotine for inhalation, along with a carrier and flavors [1]. Some e-cigs look like cigarettes that have LED lights opposite the mouthpiece (known as a "cig-alike"), some have replaceable cartridge or refillable tanks, and others are hookah-like or have vessels where users term this "vaping". (The SREC is a pod system that prevents direct exposure to e-liquids.) They are battery powered and have electronic heating elements that aerosolize carrier liquids containing nicotine. The carriers are vegetable glycerol (VG; aka glycerin) and/or propylene glycol (PG). The use of e-cigs and similar products is rapidly rising, with sales totaling more than $3.7 billion per year, and all the major tobacco manufacturers are marketing these products [2]. Fifty percent of adult smokers have tried e-cigs, and 23% are current dual users [3-6]. For adults who use multiple tobacco products, the most common combination are cigarettes and e-cigs [5]. Why adults use e-cigs vary, and include cessation attempts, health concerns and convenience to avoid indoor smoking bans [7]. The use of e-cigs has promoted much controversy among the public health community [1, 8-16]. Most professional organizations are cautious about the benefits and risks of e-cigs [17-20]. Important to the controversy is the lack of data regarding toxicity of e-cigs in comparison to tobacco smoke. Today, there is a robust public health need for studies on e-cig product appeal, use behavior and toxicity, which are challenged by the available diversity of products and flavored e-liquids [10, 17, 21]. Although there is an absence of data, there is a growing perception among lay adults that e-cigs are as risky as cigarettes [21-23].

The FDA Center for Tobacco Products has recently deemed regulatory authority for e-cigs, and their current research priorities include the study of e-cig toxicity [1]. However, some have voiced concern that increased regulation too soon would hinder an emerging market with a promising positive health impact, and also impair long term observational research needed to assess the risk of e-cigs use at the population level [24]. To make sound policy about e-cigs, regulators need data about toxicity in humans, particularly as it might affect smokers and former smokers who will regularly use e-cigs long-term [25]. To assist in this process, the National Institute on Drug Abuse (NIDA) has recently developed a Standardized Research E-Cigarette (SREC) to provide standardized exposure conditions to assess risk reduction for smokers using e-cigs. This is a critical step for toxicity studies because currently marketed e-cigs are not standardized, have unknown product performance

standards and are subject to market and manufacturing changes without notice. The SREC will be available for human studies. OSU is one of only 3 universities who will be provided the SREC for testing.

Human clinical trials provide valuable information regarding how tobacco products are used, their efficacy for smoking cessation, actual toxicant exposure and risk for disease [26-28]. In this study, we propose to conduct a clinical trial of smokers who will use the SREC (with or without nicotine) for 10 weeks, completely substituting them for cigarettes and compare them to either continued smoking (controls) or smokers who quit with nicotine replacement therapy (NRT). The subjects will undergo serial lung biomarker assessments by bronchoscopy to assess the potential for reduction of inflammatory effects of the SREC. It is expected that e-cigs generally will reduce the inflammatory effect of smoking, but less than completely quitting. While this study focuses on inflammatory responses and gene pathways, the 'omics data obtained herein and the stored biospecimens will allow for the assessment of other metabolic and potential disease pathways. We will establish a biorepository for future studies where we also can compare non-invasive biomarkers to lung biomarkers.

Smoking causes inflammation in the lungs linked to lung disease and cancer [29-34]: Inflammation is considered a hallmark of cancer and chronic obstructive pulmonary disease [32, 33, 35], and smokers, before the onset of clinical disease have documented pro-inflammatory effects in their lungs [36]. Cigarette smoke activates alveolar macrophages and airway epithelial cells to release pro-inflammatory cytokines, resulting in the recruitment of infiltrating inflammatory cells from the blood to the lung. At the same time, normal protective mechanisms for adequate tissue repair by fibroblasts are hindered by cigarette smoke. Key inflammatory cytokines (e.g., TNF-α, interleukins, and interferons) and cytotoxic mediators such as reactive oxygen species, metalloproteinases and soluble mediators of cell death are induced by smoking during chronic inflammation, to promote unregulated cell proliferation, cell invasion, and angiogenesis and genomic instability [34, 37]. In experimental animals, chemopreventive agents that inhibit inflammation reduce lung tumorigenesis [38].

An understanding of the inflammatory effects of smoking on the human lung can be directly assessed through biomarkers obtained via bronchoscopy. The procedure takes 30-60 minutes, where a flexible bronchoscope is inserted into the lung through the nose or mouth, and the lung is sampled by saline bronchoalveolar lavage (BAL) and bronchial brushings. Research bronchoscopies are commonly done for healthy smokers and non-smokers to understand the effects of smoking, and are considered sufficiently safe for the research of healthy subjects [39-62]. Of note, research bronchoscopies are commonly done for COPD patients, further demonstrating the acceptance and safety for the procedure, including by us at OSU (OSU-2013C0015; Clinicaltrials.gov NCT01867242) [43, 52-55, 63-65].

Inflammatory cell infiltrates: Using bronchoscopy, it has been determined smoking increases total cell counts, macrophages, lymphocytes, neutrophils, eosinophils and basophils, by as much as 8-10 fold [45, 46, 54, 56-61, 66, 67]. For example, 132 smokers and 295 never-smokers who underwent bronchoscopy had increased numbers of inflammatory cells in BAL samples, with a dose-response depending on smoking level [39]. Bronchial biopsies show similar results, e.g., 45 asymptomatic smokers compared to never-smokers had statistical increases for neutrophils, eosinophils, mast cells, and macrophages, with means differing 2-4 fold [59]. Important evidence comes from smoking cessation studies, where 28 smokers who underwent bronchoscopy at 12 months after quitting showed decreases of inflammatory cells compared to non-quitters [40]. Directly relevant to this application, it has been reported that sputum neutrophils statistically decreased after 6 weeks of smoking cessation in three studies [68-70], although a small sputum study did not find similar results after 4 weeks [71]. Sputum macrophages decrease as early as one week in sputum after quitting [70]. Also, there are reported decreases of pulmonary macrophages and neutrophils with smoking reduction of more than 50% [72].

Inflammatory cytokines: Lung cytokines affected by smoking (e.g., IL6, IL8, IL10, IL33, TNF-α) are associated with COPD and lung cancer [32, 54, 55, 61, 73-79]. For example, a bronchial biopsy study of 45 asymptomatic smokers and never-smokers showed statistical increases for smokers of IL-8, with means that are 2-4 fold different [59]. In another study using bronchial biopsies and immunohistochemistry of 47 subjects, IL6 was associated with smoking [79]. Inflammatory cytokines, such as IL8, are higher in patients with emphysema [73]. While one study reported that there was no difference between smokers and non-smokers for IL6 and IL8 in a

cross-sectional analysis [80], a smoking cessation study showed statistically significant reductions at 12 months for IL8 [54]. The repeatability and reliability of repeated measures for BAL cytokines has been demonstrated, but it also should be noted that blood cytokines are not a good surrogate for lung cytokines [67].

mRNA expression: The differences in mRNA expression have been well described for smokers versus non-smokers, and used for the early detection of lung cancer including gene expression related to inflammation [41, 42, 50, 81-85]. Expression profiles for genes that are up- and down-regulated have been described and shown to cluster with smoking status [81]. In 16 smokers compared to non-smokers, genes were differentially expressed relevant to the pathogenesis of COPD, including those coding for inflammatory cytokines and innate immunity, response to oxidants and xenobiotics, and general cellular processes [82]. Dose-response mRNA expression changes to urine cotinine have been identified in 121 subjects who were smoking the equivalent of only a few cigarettes per day [42]. In this large cross-sectional study, pathway analysis implicated genes involved in the metabolism of xenobiotics, arachidonic acid metabolism, and oxidative stress responses.

MicroRNAs (miRNAs) are short non-coding single-stranded RNA transcripts that negatively regulate gene expression at the post-transcriptional level. There are many studies linking smoking and COPD via changes in miRNA expression. Among the most commonly implicated in COPD pathogenesis and inflammation is miR-146a, which is related to smoking [86-90]. *In vitro* studies using cigarette smoke condensate (CSC) on human bronchial epithelial cell lines up-regulates miR-101 and miR-144, which target the cystic fibrosis transmembrane conductance regulator, and also are found to be up-regulated in COPD [91]. Other changes *in vitro* include a decrease in miR-200c, related to NF-kappaB-mediated inflammation and thought to increase epithelial to mesenchymal transition (EMT) associated with tissue remodeling and cigarette smoking in COPD [92-95]. Experimental animal models for cigarette smoke exposure have identified altered expression of miR-146a, miR-92a-2*, miR-147, miR-21 miR-20 and miR-181. Both miR21 and miR-181a are involved in chronic systemic inflammation [96] and have been reported to be affected by smoking in humans and a risk factor for COPD [97]. Cross-sectional studies assessing the sputum of smokers and non-smokers identified miR-340 overexpression [98] and alveolar macrophages alter expression of miR-210, miR-150, miR-146b-3p, and miR-452 [99]. The latter miRNA targets matrix metalloproteinase-12, which is increased in the sputum of patients with COPD contributes the development of emphysema [100, 101]. In a recent study of 19 subjects in a 3 months smoking cessation trial, 34 miRNAs in bronchial brushings were differentially expressed between the smokers and baseline non-smokers, and 22 of these decreased with smoking cessation [49]. The major function of both the up- and down-regulated miRNAs was inflammation, with several targets associated with NF-kappa B pathway. There are other examples of miRNAs related to cigarette smoke and inflammation considered to be involved in COPD, such as effects in smooth muscle, fibroblasts, macrophages and neutrophils, and specific miRNA changes in bronchial epithelia of smokers versus non-smokers [86, 102]. While our primary hypothesis is that e-cigs can alter lung inflammatory pathways, but less than smoking, it should be noted that our miRNA assessment will identify other pathways involved in COPD, such as altered tissue repair and remodeling, oxidative stress, and imbalance in fluid homeostasis [86, 102].

Mitochondrial DNA (mtDNA): Genetic alterations of mtDNA can arise due to DNA repair detects, replication errors and carcinogen or mutagen exposure [103]. Given that tobacco products include a substantial number of mutagens and carcinogens, genetic alterations by tobacco products could be important indicators of toxic effects of tobacco uses in human. MtDNA is particularly vulnerable to oxidative stress than nuclear DNA (nDNA) and its genetic errors are accumulated faster than in nDNA due to the error-prone mtDNA synthesis [104-107]. Since mtDNA is much smaller than nDNA, even slight alteration of mtDNA could potentially have significant effects on the proper function of mtDNA in relation to tobacco use [108]. Some recent studies have confirmed that there are significantly high levels of oxidant reactivity and reactive oxygen species (ROS) associated with aerosols from e-cigs [109-113], indicating the potential genetic alterations by e-cig uses. Experimental in vitro studies showed that e-cig vapor, both with and without nicotine, induces nDNA strand breaks [114, 115]. Another recent study showed that e-cig aerosols and copper nanoparticles induce mitochondrial stress and promote DNA fragmentation in lung fibroblasts [113]. Another study found that e-cig flavoring aerosol exposure increased cellular DNA damage and IL-6 and IL-8 in human epithelial cell [116]. Experimental in vivo and in vitro e-cig vapor exposures demonstrated airway epithelial changes comparable to tobacco cigarettes with similar oxidative

responses [117]). Thus, there are sufficient evidences of genetic damages by e-cig vapors. However, unlike nuclear DNA, investigation of genome-wide mtDNA alteration (mutations an copy number variation) in relation to tobacco use has been largely neglected at the molecular levels by removing mtDNA in sample preparation of genome-wide profiling or by bioinformatics tool to minimize "mitochondrial contamination". When it comes to target airway epithelial cells for tobacco exposure, the nose is an attractive source of airway epithelial cells, particularly in large population studies in which bronchoscopy may not be feasible. Several recent studies have shown nasal epithelial cells as accessible surrogate for studying lower airway biology [118-123]. In our study, we will conduct comprehensive profile of the mtDNA genetic alterations at genome-wide levels and inflammatory cytokines from the study comparing smokers, NRT users and those using e-cigs. We will also examine similarity and differences of mtDNA alterations associated with tobacco uses between nasal and bronchial epithelial cells.

Lung microbiome: The link between smoking cigarettes and lung diseases such as COPD and lung cancer is well established. There is emerging evidence that lung microbiome are contributing to lung diseases. A study between healthy smokers and non-smokers also showed that although lung microbiomes are different, they are not significantly altered by smoking [124]. A second study showed that in healthy smokers and non-smokers, the lung microbiome is fairly diverse, with *Proteobacteria, Firmicutes,* and *Bacteroidetes* the dominant phyla. However, the same study also found that there is a loss of diversity found in patients with severe COPD [125]. The loss of lung microbiome diversity and microbial dysbiosis of certain bacterial species in COPD and other lung diseases was further corroborated by other studies [126-128]. Currently, there are no studies on the effect of e-cig use on lung microbiome.

DNA Methylation: DNA methylation is a well-known epigenetic marker that regulates the function of genes and affects gene expression and has been associated with lung diseases including cancer [129-132]. Chronic inflammation, including in normal tissues from persons without cancer, has been shown to lead to altered DNA methylation [133-135]. These methylation changes in pre-neoplastic tissues were associated with inflammation [133, 136, 137]. There is evidence that pro-inflammatory cytokines may affect the activity of DNA methyltransferases [138] and that cytokine expression may be controlled by DNA methylation and other epigenetic mechanisms [139]. In studies examining genome-wide changes in DNA methylation, a number of differentially methylated sites have been identified between smokers and never-smokers. Specifically, loci identified include *F2RL3*, *AHRR*, and intergenic regions in 2q37.1 and 6p21.33 [140-142]. With regard to former smokers, it appears that some, but not all, of the smoking associated changes in DNA methylation are reversible, even decades after cessation [143-145]. However, most of these studies are comparisons of methylation in blood cells. A study of small airway epithelial DNA obtained by bronchoscopy identified 1,260 CpGs (1,120 unique genes, enriched in oxidative response pathway) to be differentially methylated in COPD compared to control subjects. 158 of these unique genes including *CYP1A*, *GSTM1*, and genes involved in Aryl hydrocarbon receptor (*AHR*) signaling were significantly associated with smoking with pack years [146].

Untargeted metabolomic profiles: Metabolomics is an emerging technology that is being used to identify new biomarkers of tobacco smoke exposure (including by us) [147-155], and for studying COPD [156-158]. The assay assesses thousands of small molecules (< 1500 Daltons) that reflect exogenous exposures and cellular responses to those exposures. Metabolomics is now being widely applied to evaluate disease and disease causation [159-162]. In the case of smoking, metabolomic screening can reveal changes induced by cigarette smoke constituents as well as those due to endogenous cellular responses to cigarette smoke. We have published the usefulness of metabolomics for assessing smoke exposure in the blood and urine [147-149]. We have identified novel biomarkers related to smoking (e.g., glycophospholipids and pathways related to inhibition of cAMP) that include some that differ by gender and race, and identified the presence of menthol metabolite}. We are not aware of metabolomics studies in the lung, but we also have demonstrated the feasibility for an untargeted metabolomics assessment for BAL (see below). Other studies suggest the utility for a BAL assessment, such as those that show changes in smokers' sputum [163], and for a bronchoscopy study for air pollution [164]. In an animal model, BAL metabolomics have mapped with emphysema progression, identifying a lung specific L-carnitine as a central metabolite [165].

Cytometry by Time of Flight (CyTOF) analysis: Using a mass spectrometry technique based on inductively coupled plasma spectrometry and time of flight mass spectrometry will determine the cellular expression, properties of cells (cytometry) to distinguish inflammatory cell subtypes.

Proteomics analyses: Ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) is a well-established method to survey the proteins at a global level from a biological sample of interest. Label-free quantification and tandem mass spectrometry will be employed to assess and quantify the global proteomic profiles. Proteins from epithelial cells will be digested into peptides and separated by reversed phase chromatography prior to infusion into a Q-Exactive-HF electrospray tandem mass spectrometer. Using this approach, approximately 5,000 proteins can be quantified in each patient sample. For the data analysis, the relative abundance of the proteins will be compared in e-cigarette samples versus control samples. Individual peptides will be identified by their tandem mass spectra and correlated with their associated proteins. The abundance ratios of the peptides enable relative quantification of the associated proteins. These values can then be used to determine proteins that are differentially regulated. Ingenuity Pathway Analysis, a software designed to map the proteins to known signaling pathways, will also be used to gain a better understanding of the changes that e-cigarette use has on cellular functions.

Nitric oxide: Fractional exhaled nitric oxide (FeNO) is a validated marker of lower airway inflammation that is simple to assess, non-invasive and reproducible [166, 167]. It is used for the diagnosis and treatment of asthma in children [168-172]. Nitric oxide (NO) is present in cigarette smoke and synthesized in the lung by NO synthase (NOS) and the oxidation of L-arginine to L-citrulline [32]. The inducible NOS (iNOS) is transcriptionally regulated by pro-inflammatory cytokines in epithelial cells and macrophages in the airways [173]. FeNO is decreased by almost 50% in smokers [174-177]. The reduction in FeNO also is thought to be related to nitric oxide synthase inhibition due to cigarette smoke carbon monoxide and/or oxygen free radicals [175, 178]. Reduced FeNO has been reported to be significantly associated with neutrophilic inflammation [179]. It has been reported that FeNO reverts back to non-smoking controls levels after smokers quit for 4 or 6 weeks [69, 71]. In this application, FeNO will be used as a non-invasive marker for the reduction in inflammatory related to e-cig use.

Summary for the effects of combustible cigarette smoking on the human lung: Numerous studies have implicated changes related to inflammatory responses related to smoking. Critical pieces of evidence include the demonstration of differences between smokers and non-smokers, a dose-response with smoking, and reversal of changes with cessation. Given that there is sufficient evidence overall to consider that smoking has an effect on inflammation, it is reasonable to consider that other inhalational exposures also will induce an inflammatory response.

We are planning to conduct a 10 week trial on product (2 week run in period and 8 week intervention). The rationale for this duration balances data showing reversibility for many of the above markers within 4-6 weeks versus a potential concern for retention of smokers in a trial for longer periods of time using an untested product; SREC acceptability to smokers is unknown. Although a longer trial might reveal additional e-cig related changes, 8 weeks should be sufficient to provide meaningful data because, as summarized above: 1) it is known that lung inflammation following infection usually reverses by 6 weeks detected radiographically; and 2) sputum macrophages decrease within 1 week [70], FeNO decreases to normal within 4-6 weeks [69, 71], neutrophils decrease within 6 weeks studies [68, 69], and many gene expression associations reverses within three month of cessation [49]. For this study, we will monitor inflammatory cell counts in real time for each of the groups to assess reduction in cell counts.

Viral exposure precautions: As COVID-19 or other viral illnesses persist, study procedures have been updated to adhere to institutional standards and best practices. Given the situation continues to evolve, study procedures may also adapt to address subject and personnel safety as well as study and institutional resources. For example, the study may be temporarily suspended if there is significant community spread and resources (e.g., bronchoscopy staff or space) are limited. Similarly, as vaccination uptake increases or if scientific evidence demonstrates prolonged immunity after exposure, testing prior to procedures may not be necessary. Regardless, staff will implement sufficient precautions to mitigate viral exposure. Such precautions may include limiting

physical interactions, when possible (e.g., remote/telephone visits or physical in-person distance), assessing signs and symptoms of viral illness (e.g., temperature and exposure assessments), and providing appropriate personal protective equipment (PPE) to subjects and staff (e.g., masks, gloves, googles, face shields, etc.). While these conditions may vary throughout the study, institutional standards will be followed. Additional details are provided in the Methods section.

What is known about e-cig aerosols that might be toxic: While there are numerous recent reviews for the risks and benefits of e-cigs, there are substantial research gaps in our knowledge [16, 21]. E-liquids are composed mostly of PG, VG, and flavorants. When used in foods and skin products, these constituents are "generally regarded as safe" by the FDA [110, 180]. However, it is unknown what happens to the lung when these constituents are heated and inhaled. In e-cigs, PG can be converted to propylene oxide [1, 181], which is an irritant and an International Agency for Research on Cancer group 2b carcinogen [182]. Heated VG and PG can be converted to acrolein, acetaldehyde and formaldehyde, which also are known strong irritants and affect inflammation [183-185]. The aerosol constituents are not limited to these, however. One study identified 31 chemical constituents in e-cig aerosols, including glycidol, acetol, and diacetyl [186]. There are additional reports demonstrating the presence in e-cig aerosols of these and other potentially harmful chemicals, including tobacco specific nitrosamines (TSNAs), aromatic hydrocarbons, acetone, and volatile organic compounds (VOC) (e.g., benzaldehyde, propionaldehyde, crotonaldehyde) [1, 21, 185, 187-204]. A recent study using mass spectroscopy identified over 115 VOCs, many that were not present in the unheated liquids [188], while another identified trace quantities of benzene, methyl ethyl ketone, toluene, xylene, styrene, and acetic acid [205]. However, their presence is substantially less than for cigarette smoke.

There has been some toxicology testing for e-cig liquids and aerosols, but these are limited and the relationship to human disease risk is unclear [8, 206, 207]. Existing studies suggest that the toxicological responses are qualitatively similar to smoking, e.g., exposing cell lines and culture to the aerosols induce an effect on inflammation [208, 209], disruption to epithelia barriers [210], oxidative stress [211], cytotoxicity [212], neutrophil inflammatory response [213] and DNA damage [114, 115]. However, the magnitude of effect is low compared to cigarette smoke. Aerosols were not found to be mutagenic [214]. Normal human bronchial epithelial (NHBE) cells exposed to e-cig aerosols, with or without nicotine, increase IL-6 and IL-8 cytokine levels [215]. Another study reported a change in the gene expression pattern of NHBE cells with silenced p53 and activated KRAS when exposed to e-cig aerosol [181]. Separately, e-cig liquid was assessed in NHBE cells in parallel with a knock-out mouse model; there were increased rates of infection, inflammatory markers and altered gene expression [216]. Metals present in e-cig aerosol are capable of causing cell injury and inflammatory cytokine induction, e.g., in human lung fibroblasts [113]. There have been some studies of gene expression in cultured human bronchial epithelial cells showing changes in profiles that are much less than smoking but clearly distinctive [217]. The pathways that have been implicated in these studies include phospholipid and fatty acid triacylglycerol metabolism, with enrichment of cell cycle associated functions (e.g., cell cycle checkpoint regulation, control of mitosis) and immune system function.

In vitro studies using human bronchial epithelial cells demonstrate that increasing voltage decreases cell viability and increases the release of inflammatory cytokines (IL-1β, IL-6, IL-10,CXCL1, CXCL2 and CXCL10) [218]. Experimental animal studies also have shown that there are some toxic effects in the lungs of e-cig aerosols, which includes pro-inflammatory responses [8, 207, 219]. While *in vivo* studies indicate that aerosolized PG or VG alone only have slight toxic effects in the lung [220-223], more recent data using e-cig devices are identifying various effects on inflammatory and other responses. For example, mice exposed to e-cig aerosols showed increased lung macrophages, neutrophils and lymphocytes, with or without nicotine [215]. Separately, mice exposed to e-cig aerosol intratracheally had an increased rate of inflammatory infiltrate and cytokines, and IgE production [224]. Other studies show lung oxidant reactivity and reactive oxygen species increasing inflammatory cytokines (i.e., increasing IL-8), changes in lung fibroblasts thought to be part of COPD pathogenesis and altered redox balance [111]. A recent animal study showed measurable effects on inflammation and lung injury for both cigarette smoke and e-cigs, but much less for the latter [209].

In humans, acute health effects from e-cigs are minimal and short-lived [20, 225-232]. The most common adverse effects reported across studies were nausea, headache, cough, and mouth/throat irritation, which were similar or less compared to nicotine patches. Although adolescents using e-cigs reported an overall increased rate of chronic bronchitis symptoms [233], smokers with COPD who switch to e-cigs had a reduction in symptoms and an improved quality of life [234, 235].

A detailed summary about e-cigs and the public health consequences has just been published by the National Academy of Medicine, under request from the FDA (http://nationalacademies.org/hmd/Reports/2018/public-health-consequences-of-e-cigarettes.aspx). In this report, the authors wrote on page 21-1: "Based on the findings of this report, e-cigarettes cannot be simply categorized as either beneficial or harmful to health. The net public health outcome depends on the balance between adverse outcomes (increased youth initiation of combustible tobacco cigarettes, low or even decreased cessation rates in adults, and a high-risk profile) and positive outcomes (very low youth initiation, high cessation rates in adults, and a low-risk profile). In some circumstances, adverse effects of e-cigarettes clearly warrant concern, such as the use of e-cigarettes among non-smoking adolescents and young adults, devices that are prone to explosion, and the presence of constituents in e-cigarette liquids that are of major health concern (e.g., diacetyl and some other flavorings). In other circumstances, namely regular combustible tobacco cigarette smokers who use e-cigarettes to successfully quit smoking, e-cigarettes may represent an opportunity to reduce smoking-related illness. For these reasons, e-cigarette regulation that merely considers whether to be restrictive or permissive to the marketing, manufacture, and sales of all e-cigarettes for all populations is unlikely to maximize benefits and minimize the risks."

Clinical studies and case reports have studied the acute health effects of e-cigs on humans, which until recently have been considered mild [236-239]. However, while there have been scattered case reports over the last 5 years of serious pulmonary toxicity [240-243] (https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html), a relationship to e-cig use has been unclear. In the past 6 months, clusters of cases are now appearing across the U.S., and have included deaths. The causes are under active investigation and so the nature of the respiratory illness is unclear, and to date no single device or contaminant has been found. The majority of patients have reported vaping cannabis oil, such as THC, obtained on the black market or other sources. In addition, one of the chemicals that has been possibly linked to the addition of is Vitamin E acetate to e-liquid cartridges and pods (tocopheryl acetate). Many of the respiratory illnesses resemble lipoid pneumonia and laboratory studies suggest disorders of lipid homeostasis [244]. Bronchoscopies of healthy e-cig users also point to altered lipid homeostasis and matrix proteins [245, 246]. These clusters of cases have now led the FDA and CDC to advise consumers to protect themselves by not starting to use e-cigs and especially not using vaping products of any kind obtained off the street or black market and to refrain from using THC oil or modifying/adding any substance to vaping products purchased at stores. (Our study will now include explicit warnings about this, including the advice to not alter any commercial products.) Explicit recommendations by the FDA and CDC have not advised smokers to stop using commercial and unaltered e-cigs. Investigations continue to focus on adulterated products and contaminants. Also, whether these illnesses represent idiosyncratic and rare reactions or are bell-weather responses to a more general and long term chronic health effect is unknown. (And needs to be considered in the context if e-cigs can help smokers quit, albeit risks that exist much like any medical treatment including smoking cessation drugs.) The implication is that these new reports represent some recent change in devices or how they are used, and that existing commercial products are not associated with these serious and rare respiratory effects. Thus, studies such as this protocol have the potential to provide important information about health e-cig users.

Important information about potential toxic exposures from e-cigs can be learned from human biomarker studies, which show that there are substantial reductions in exposure for complete switching. All of the published studies that we are aware of, however, use peripheral biomarkers (e.g., urine and blood) or exhaled air, and not those collected directly from the lung. A recent study by Cravo, et al. (2016) randomized 419 smokers to an e-cig or continued smoking over 12 weeks [229]. (Recently published studies are important because they provide data for the most advanced generation e-cigs.) They found statistically significant decreases for the e-cig users compared to the controls for urinary metabolites of acrolein (3-HPMA), benzene (S-PMA) and NNAL (a pulmonary carcinogen). Another important measure in that study was urinary PG, which almost doubled after

one month of e-cig use (the product used was 75% PG; we propose to measure urinary PG to assess e-cig use). For another recent study (2016), albeit much smaller, of 20 smokers switched for only two weeks, there were reductions for a large panel of biomarkers, including a 50% reduction in acrolein metabolites (CO, NNAL and all measured VOCs and PAHs) [247]. McRobbie and coworkers (2015) reported that among 40 smokers there was a statistically significant decrease in acrolein exposure after 4 weeks [248]. Pulvers and co-workers (2016) studied 40 smokers and reported substantial reductions to non-smoking levels for urinary NNAL, but only for 2 of 8 VOCs, namely benzene and acrylonitrile [249]. Carbon monoxide (CO) also was substantially reduced. O'Connel and coworkers (2016) reported on a five day trial of 105 subjects confined to a clinical facility, and similar reductions in the urinary biomarkers and CO were reported [250, 251]. Last, a one year clinical trial demonstrated significant reductions in exhaled CO [252]. The measurement of FeNO among e-cig users has been reported in four studies showing a decreased FeNO [250, 252-254] (including 1 trial that was of 1 year duration), while another found no difference [255], and another with methodological limitations reported an increase (e.g., e-cigs and controls were tested on different days) [256]. In this application, we will assess FeNO in order to establish a relationship for this marker with the lung markers to determine if it can be a valid surrogate for lung effects.

Nicotine is a highly bioactive agent that inhibits inflammation: This study will include an assessment of the SREC with and without nicotine. Nicotine content can be regulated by the FDA and some considerations for this will be affected by the addictiveness (abuse liability) of the product, but toxicity considerations may also apply. It is well established that nicotine is highly bioactive in that it induces proliferation, inhibits apoptosis, promotes the epithelial to mesenchymal transition (EMT), and promotes angiogenesis [257, 258]. All of these are important components of cancer and COPD [219, 257]. To date, nicotine is not considered a significant carcinogen for humans as nicotine replacement therapy or in low-TSNA smokeless tobacco (snus) have not demonstrated increased risks of cancer [259]. Regarding inflammation, nicotine is both pro- and anti-inflammatory, and therefore theoretically able to affect cancer and COPD pathogenesis in different ways [258, 260-265]. In cell culture studies of human bronchial epithelial cells, while cigarette smoke condensate increases inflammatory cytokine production, nicotine does not, and pretreatment with nicotine reduced the condensate effects [261]. In a study of wound healing in smokers, compared to continued smoking and quitting with or without nicotine, it was observed that the nicotine replacement therapy (NRT) reduced inflammation and macrophage infiltration, but not angiogenesis [260]. In human nasal epithelial cells, in contrast to cigarette smoke and acrolein, nicotine induced inflammatory cytokine response [266]. *In* vivo, nicotine was able to inhibit acute lung injury in mice through anti-inflammatory effects [265]. The anti-inflammatory effect may be through the stimulation of nicotinic receptors present in lung and other cells, and there are data that nicotinic receptor agonists reduce acute lung injury [262, 267, 268]. There are nicotinic receptors on macrophages that reduces pro-inflammatory cytokines while having no effect on anti-inflammatory cytokines [269]. In contrast to data for nicotine reducing inflammation, other data, using different experimental models, indicate that nicotine may increase inflammatory response because of its toxic effects on the lung epithelium [210, 216]. Pro-inflammatory effects have been observed in cell culture models of vascular smooth muscles and in atherogenesis, because it can induce oxidative damage [270, 271]. In contrast to the above cited studies, some data indicate that nicotinic receptors increase inflammation in human lung and decreases lung function [267]. Because of the potential anti-inflammatory effect of nicotine, it has been tested if NRT can be used as a treatment for inflammatory disease, such as ulcerative colitis, but the results have been inconclusive to date [264, 272]. In this application, we will test the SREC with and without nicotine providing a unique opportunity to assess nicotine effects on the lung in the context of e-cig aerosol exposure, an important research gap [219]. For the purposes of this study, our hypothesis is that nicotine will attenuate an e-cig aerosol inflammatory response because most of the data supports a greater anti-inflammatory effect and human studies do not indicate that NRT is a carcinogen. Future studies may consider exposures in humans with e-cigs having differing levels of nicotine, but the first step to demonstrate an effect by nicotine would be the comparison for the most extreme difference, i.e., with and without nicotine, as proposed herein.

Summary of Significance: This study is significant in the following ways:

- This study will directly assess the inflammatory response of the SREC aerosols in the human lung for smokers who use e-cigs for 8-week on-product experimental condition, compared to continued smoking or

quitting with NRT. The lung is an important target organ for smoking-related disease, i.e., cancer and lung disease.

- The focus on smokers is particularly significant in that about 50% of smokers are known to have tried e-cigs and about 23% of smokers have switched to e-cigs as cessation devices, and will compare the reduction in inflammation compared to known and effective ways to quit smoking [3-6]. This data is relevant for informing smokers about their choice of how to quit smoking.
- Nicotine content and delivery can be regulated by the FDA. The pulmonary effects of nicotine inhalation are essentially unknown, but it is plausible that it has an effect because it is a highly bioactive molecule. The SRECs provide a unique opportunity to provide data for the magnitude of such an effect, if any.
- This study will use the SREC. It was developed by NIDA because of an important research gap regarding e-cig toxicity, where essentially no two commercial e-cigs are alike, and their product performance and toxicant yields are generally unknown. These likely differ among the diverse products on the market. Also, commercial products frequently change in their marketing and performance without notice (e.g., a product may be withdrawn from the market) making ongoing studies problematic. These issues are addressed by the NIDA's development of the SREC, and long term availability.
- Assessing the SREC's effect on pulmonary toxicity allows for the comparison of studies conducted by other investigators who also use the SREC in both human and preclinical studies.
- In addition to examining SREC, this study may examine the effects of other devices, such as Juul and IQOS (and potentially other novel products) on pulmonary toxicity.
- The proposed application will establish the feasibility for use of the SREC for clinical trials using bronchoscopy, and provide baseline data to design future studies of commercial e-cigs.
- This study will utilize biomarkers of diverse physiological responses, all relating to inflammation, namely combining gene expression, metabolism, cell infiltrates and inflammatory cytokines. Importantly, the multi-omic approach will allow us to examine other disease pathways, including those affected by nicotine.
- The infrastructure for conducting e-cig and smoking-related bronchoscopy studies is established. Our preliminary data indicates that we can accomplish this study (see below). The OSU IRB protocol is approved to conduct serial bronchoscopies in healthy never smokers trained to use nicotine- and flavor-free e-cigs (OSU-2015C0088; Clincaltrials.gov NCT02596685) and accrual is going briskly. We also have a separate infrastructure to conduct multi-arm randomized trials for smoking behavior and biomarkers (OSU-2013C0015; Clinicaltrials.gov NCT01867242).
- This study will include a biorepository of urine, blood, saliva, lung and nasal samples for future studies to compare non-invasive biomarkers with lung biomarkers.
- This study was funded by application responding to NIDA PAR-17-156: Evaluating the NIDA Standardized Research E-Cigarette in Risk Reduction and Related Studies (U01) because we are studying the health effects of e-cig aerosols in the human lung using the SREC. The research objective for the PAR was: "This FOA will encourage short studies that can be completed within a shorter time frame. Applications submitted to this FOA should use subjects who are current smokers and investigators should use the NIDA SREC in their studies." The PAR specifically encourages the following research: "Health effects associated with changes in toxin exposure as a result of SREC use compared with conventional smoked tobacco". It also will provide data for: "The ability of conventional smoked tobacco users to switch to the nicotine-containing or placebo SREC and the impact on health risk endpoints." Our application was only one of 3 studies funded by NIDA with an impact score of 17.

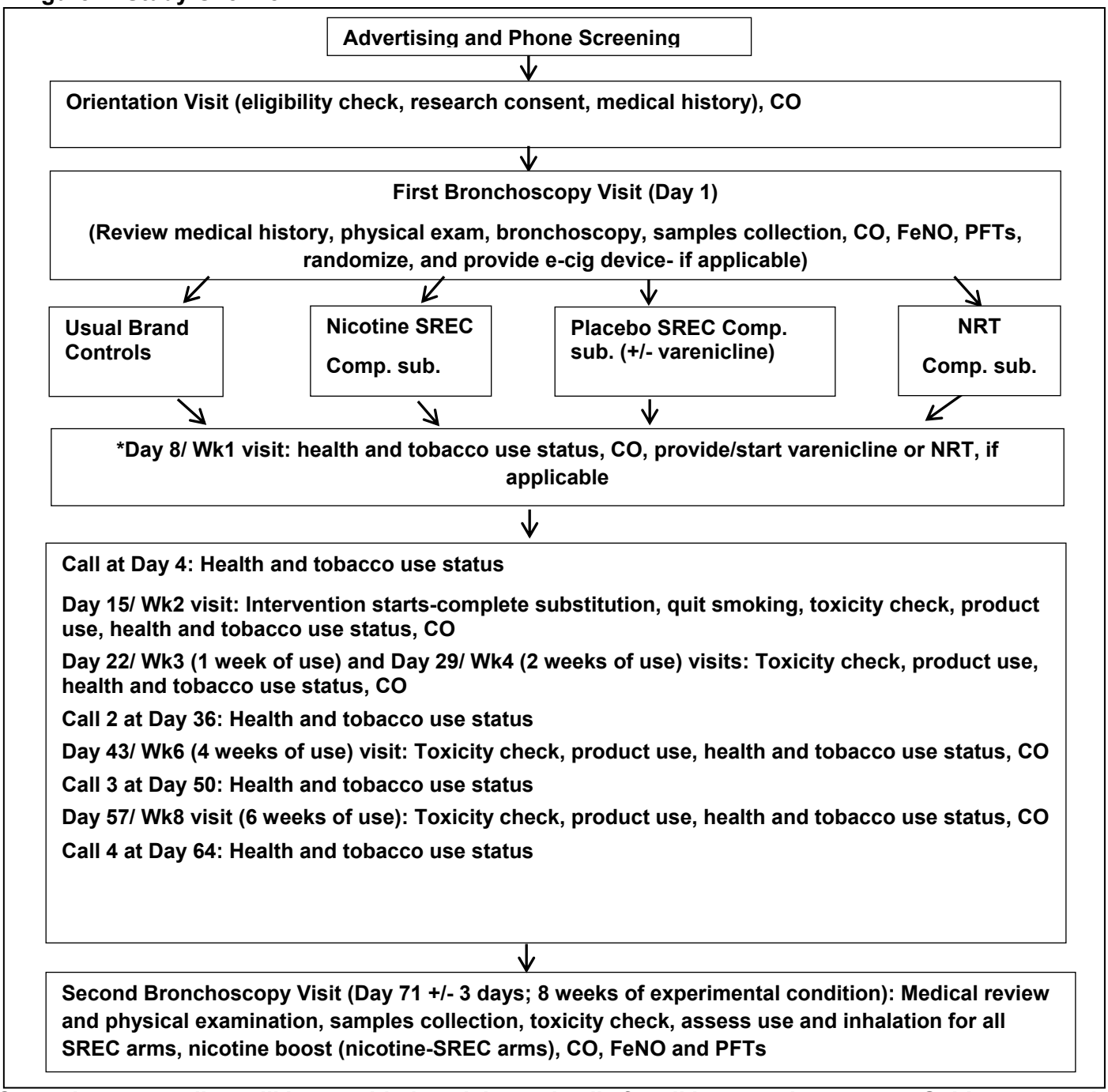
INNOVATION

- This study will novelly assess the effects of the SREC (and potentially other newly available product) aerosols directly in human lungs of smokers who completely substitute the SREC for smoking, and compare this to quitting with NRT.
- The extended use over 10 weeks (8 week intervention) on product that will allow for the subject to learn how to use the product and adjust to adverse effects for two weeks (if any) that are generally short-lived, and allow the lungs to respond over 8 weeks.
- Conducting a trial with serial bronchoscopies before and after the use of the SREC device allows for within-subject comparisons as well as including comparisons to a randomly assigned group.

- The use of multiple endpoints for gene expression (mRNA and miRNA), metabolomics, cell and cytokine changes are comprehensive and complementary. This allows for an integrated assessment for multiple ways that inflammation can happen to distinguish treatment effects.
- This study uses 'omics to assess inflammation, which also provides data for other disease pathways.
- This study will provide new data for the effects of nicotine on pulmonary inflammation using a device demonstrated to deliver nicotine at levels similar to cigarettes.

4. Methods

Figure 1: Study Overview



***Cessation counseling will be provided at visits and calls for all intervention groups. Subjects may also proactively contact study staff for additional support.**

a. Study Design

i. Study arms/groups

Participants will be randomized to one of four groups of subjects (n=32 for each group): controls who will continue to smoke their usual brand, a group who will switch completely to the SREC with nicotine, a group who will switch completely to the SREC placebo, and a group who will stop smoking using nicotine replacement therapy (NRT).

ii. Randomization process

Participants will be randomized to one of four groups. This design allows for a rolling admission and increased contemporary controls in the event a device is not available at the time of enrollment. The conditions subjects are randomized to may vary depending on product availability. Within each group, we will also randomize the patients to sequence the right or left lung for the baseline bronchoscopy in an approximate 1:1 ratio. These patients will then receive a second bronchoscopy in the opposite lung at ~71 days in order to reduce the influence of the baseline procedure at follow-up. The randomization procedures will be supervised by the biostatistician.

iii. Intervention type/process

This study is a 10-week clinical trial (8 weeks on the experimental condition after a scheduled quit date; n=128) for healthy smokers randomized to one of four groups. The duration for the intervention period has been extended from 4 to 8 weeks to harmonize with two other NIDA grantees who are studying the SREC, at the request of NIDA. An overview is shown in **Figure 1.** Each group is 32 subjects. All subjects will undergo a baseline bronchoscopy. Bronchial brushings and BALs will be collected during the bronchoscopies to assess total and differential cell counts, inflammatory cytokines, mRNA gene expression, miRNA expression, genome methylation, microbiomes, proteomics, CyTOF, pulmonary function tests and BAL metabolomics. Subjects also will be assessed for THC exposure, exhaled FeNO, exhaled CO, and urinary PG and glycerin.

At the baseline bronchoscopy visit, subjects will be randomized to either one of the four groups (control and interventions). The SREC intervention groups will be instructed on how to use their assigned product and after the visit begin to use the device daily and will be allowed to continue smoking, in order for them to learn how to use the device. They will be told that after two weeks of use to begin their assigned condition, essentially establishing a smoking quit date (e-cig-only) on day 15. To mediate withdrawal symptoms and increase retention, subjects assigned to SREC placebo group will be offered varenicline at no cost, and used according to FDA indications. Those participants will be given the medication at the day 8 visit, one week before the day 15 quit date. The NRT group will begin to use the NRT one week before the day 15 quit date. This design allows for a rolling admission and increased contemporary controls in the event a device is not available at the time of enrollment. The conditions subjects are randomized to may vary depending on product availability.

After 10 weeks of their assigned condition (+/- 3 days), subjects will undergo a second bronchoscopy as described above. Subjects assigned to the nicotine-SREC or placebo-SREC conditions will undergo inhalation assessment. In an effort to standardize the timing and recent product exposure, subjects be instructed to use the device immediately prior to the visit. Subjects who report the device was not used as instructed will be asked to use the device before proceeding with the visit. Subjects assigned to the nicotine-SREC condition will also undergo a nicotine boost assessment that includes two blood draws, one prior to and one after a timed use session.

Subjects assigned to the control condition (smoking their usual brand) will undergo all the procedures as the other conditions, except the nicotine boost and inhalation assessment. The specific value using controls to users, rather than only assessing within subject changes for pre- to post-bronchoscopy biomarkers, is our ability to evaluate the impact of being enrolled in a smoking study, e.g., smokers in the control group might reduce their exposures because they are enrolled in a smoking study.

A biorepository of the lung samples, saliva, urine, blood, nasal lavage and nasal brushings will be established. A Data and Safety Monitoring Board will be established as described below.

b. Participant Selection

Eligibility Criteria

- Male or female subjects who are age 21-52*;
- Smokers who smoke ≥5 filtered cigarettes/day for ≥1 year*;
- No unstable or significant medical conditions as determined by medical history (see exclusion criteria below) to ensure safety of the subject, to minimize the effects of poor health on biomarker measures and to maximize compliance to study procedures);
- Negative COVID-19 test (if applicable)**;
- Subject has an electronic device and is willing to download the iCO app to report exhaled CO level daily;
- Able to read adequately to complete the survey and related study documents or give consent; and,
- Subject has provided written informed consent to participate in the study.

Exclusion Criteria

- Regular consumption of hand rolled cigarettes (i.e., a machine is not used to standardize the process);
- Immune system disorders requiring medication;
- Prior diagnosis of chronic pulmonary disease (e.g., asthma with regular use of medications, COPD, chronic bronchitis, and restrictive lung disease);
- Acute bronchitis or pneumonia within 1 year;
- COVID-19 diagnosis or related symptoms within the last 3 months**;
- Reported history of diagnosed kidney or liver disease;
- Any medical disorder that will increase the risk from bronchoscopy, affect biomarker data (i.e., cancer), or increase risk of an adverse effect from e-cig use;
- General anesthesia within 1 year;
- Regular use of inhalant medications in the last 3 months;
- Use of antibiotics in prior 30 days;
- Use of steroids, including corticosteroids, in prior 30 days;
- Allergies to study medications, such as, lidocaine, Versed, Fentanyl or Cetacaine;
- Allergies to propylene glycol/ glycerin or flavors;
- History of hypersensitivity to varenicline;
- Bronchoscopy or any other lung procedure for any reason within the previous 6 months;
- Current or recent (within three months) alcohol or drug abuse problems;
- Regular use of an e-cigarette or other combustible tobacco products in the prior 3 months (not to exceed 10 times and no use within 2 weeks) - subjects will be asked to refrain from using marijuana and all other tobacco products while active in the study (however, use is not an exclusion);
- Currently using nicotine replacement or other tobacco cessation products (to minimize confounding effects of another product) or intention to quit in next three months;
- History of bronchospasm or adverse reaction to previous e-cig use;
- BMI > 40 (risk of unstable airway);
- Pregnant or breastfeeding in prior 3 months- If the subject is female, a urine pregnancy test at no cost to the subject will be done on the day of bronchoscopy***;
- Subject does not have an electronic device or is unwilling use the iCO app to report exhaled CO; and
- Unable to read for comprehension or completion of study documents.

* Age and number of cigarettes smoked per day increases the participant pool and generalizability.

** According to institutional standards, which may evolve throughout the study, if a subject self-reports testing positive for COVID-19, the condition should be resolved without ongoing symptoms for at least three months. If a subject tests positive before the first bronchoscopy, the subject would be placed on a wait list for at least three months and re-tested before his/her appointment; if a subject tests positive before a follow-up bronchoscopy, his/her participation will be withdrawn by the PI.

*** All females of child-bearing potential (i.e., not postmenopausal or surgically sterile) will provide a spot urine sample to be used for a urine pregnancy test. Research staff will read the test result and document positive or negative in the CRF. All females of child-bearing potential must agree not to become pregnant and to use an approved form of birth control (birth control pills, implants, IUD, Depo-Provera, or double barrier method (e.g. condoms and diaphragm)) while participating in the study.

All subjects are screened by a pulmonologist obtaining a medical history and a physical examination (heart, lungs and oral cavity) to ensure no increased risk from bronchoscopy or e-cig use.

i. Recruitment Procedures

Subjects will be recruited from advertisements through a variety of media outlets and the internet, as well as community events, and including Research Match, Study Search and a registry of subjects interested in future tobacco studies, such as (2014C0030). This latter important source for identifying subjects is currently conducting a mailing to 170,000 homes in Franklin County and southern Ohio to identify subjects for three of the current P50 projects (OSU 2014C0030); families who return the mailings agree to be contacted for future studies, and the returned surveys will identify smoking status. Thus, for subjects not selected for existing P50 projects, they can be contacted for this study.

Subjects will also be recruited through respondent-driven sampling (RDS), which is commonly used in studies involving contact tracing and social networks (e.g., sexual health studies). This sampling method is a type of peer-driven chain-referral sampling (Heckathorn 1997, 2002). Although there are biases associated with chain-referral sampling that can affect the composition of the sample achieved, RDS can control these biases through its methods of data collection and analysis. RDS initial recruiters who are study subjects will recruit future subjects (seeds) who in turn recruit other subjects (seeds). This chain of recruiters and recruits then continues for multiple “waves” of recruitment. Ongoing recruitment is fostered with a dual incentive system: one incentive for participating in the project and another incentive for each person recruited. Recruiters are linked to their seeds by a unique number or code on recruitment coupons/flyers, and they are limited in how many people they can recruit. The identity and status of individuals who may have provided RDS codes/coupons will not be disclosed. The seeds who meet the basic criteria (current smoker, age 21 or older, willing to be screened) can choose to participate in the study or not. Referring individuals to the study is optional. Subjects’ participation in the study will not be affected if they do not invite others or if those they may invite do not meet the criteria outlined above.

The various approaches used for this study will foster recruitment across a spectrum of education and socioeconomic status, and race/ethnicities.

The advertisements would read as follows:
“*Cigarette smokers (age 21-52) are needed for a research study to help understand the effects of using electronic cigarettes on the lungs. Volunteers should be willing to undergo two medical procedures called a bronchoscopy. Volunteers may be asked to continue to smoke their own cigarettes, use electronic cigarettes or quit smoking using nicotine replacement therapy. Volunteers may earn over $1,100 for completion of the study.”*

Advertisements may also include the following statements:
* Marijuana users will be permitted to enroll in the study
* Volunteers will not be excluded for using any form of marijuana (e.g., smoking marijuana).

Information Warehouse: Subjects will also be recruited using data from the Information Warehouse (IW) to identify potential participants and providers. The IW will be petitioned to query the electronic medical records for patients seen within the last 12 months by an OSU provider. Subjects will be:

- Current smoker;
- Age 21-52;
- Absent of medical conditions (COPD, chronic bronchitis, restrictive lung disease, kidney or liver disease);
- BMI <40;
- Live in Columbus or surrounding areas (Counties: Franklin, Delaware, Morrow, Marion, Union, Hardin, Logan, Madison, Champaign, Clark, Madison, Fayette, Pickaway, Ross, Fairfield, Hocking, Perry, Licking, Muskingum, Coshocton, and Knox).

The IW request will also include the patient's provider and the patient's contact information and their EMR number to track the patient's vital status.

The PI will contact providers identified by the IW asking for their permission to send a dually signed recruitment letter introducing the study. Upon receiving provider permission, study staff will prepare the letter and send to the patient. The provider will not be engaged in research. His/her involvement will be limited to allowing study staff to send a recruitment letter after provider approval. Study staff will send the initial letter and a follow-up letter approximately 30 days after if no contact is initiated. The letter(s) may also be sent electronically if an email address is provided by the IW. Two versions of the initial recruitment letter will be available based on the provider's preference: 1) patients can opt out and study staff will wait at least 7 days after the letter is mailed to contact the patient by phone; or 2) patients can opt in and study staff will only call patients who initiate contact.

My Chart: In addition to the IW, this study may recruit through My Chart. Subjects will be:

- Active MyChart users;
- Smokers;
- Men and women;
- Age 21-52;
- Absent of medical conditions (COPD, chronic bronchitis, restrictive lung disease, kidney or liver disease);
- BMI <40;
- Live in Columbus or surrounding areas (Counties: Franklin, Delaware, Morrow, Marion, Union, Hardin, Logan, Madison, Champaign, Clark, Madison, Fayette, Pickaway, Ross, Fairfield, Hocking, Perry, Licking, Muskingum, Coshocton, and Knox).

Social Media: Social media platforms such as Facebook, Instagram, YouTube, etc. will be used to recruit users from Columbus and surrounding areas (up to 100 miles) ages 21-52 years interested in smoking. OSUCCC Marketing will host a landing page linked to the social media videos. Ads will be linked to a landing page (http://go.osu.edu/srec-study). Those interested in the study may directly contact study staff or request more information by completing a short questionnaire and providing contact information, age, and number of cigarettes smoked per day. An automatic email will be sent to the study team who will contact the subject to provide more information about the study and schedule an orientation visit as needed. As part of their routine tasks, OSUCCC Marketing will monitor the account including the SREC study posts daily and respond to any comments. When responding to comments, OSUCCC Marketing will direct users to the landing page or study personnel to answer questions.

ii. Informed Consent Process

If subjects meet the eligibility criteria for the study, they will be given the option to review the study details with the study staff remotely or in-person at the orientation visit.

Remote Study Review: Study staff will review study details and key aspects. The PowerPoint may be used to facilitate the discussion. The ICF may be provided for the subject to read prior to the in-person orientation visit. At the orientation visit, subjects who reviewed the study remotely will be asked if they would like to review any of

the study details and if have any questions. All questions will be addressed, and the subject will be given as much time as needed to review the ICF before signing.

In-person Study Review: Subjects who do not review the study remotely will do so in-person at the orientation visit. The PowerPoint may be used to facilitate the discussion. All questions will be addressed, and the subject will be given as much time as needed to review the ICF before signing.

Subject signature may be captured as a wet signature or electronically (adhering to institutional policies). The participant will be given a copy of the document for his or her records. Eligibility will be confirmed through study questionnaires (administered after consent is provided) and reviewed by the pulmonologist and PI.

Prior to any study visit, study staff will contact subjects to ask about possible COVID-19 symptoms.

iii. Risk and benefits

Information on risk and benefits is provided in the Human Subjects section below.

c. Study Procedures

i. What will be done

Study staff will include Investigators or key personnel, identified with the study, or clinical staff associated with the pulmonary department. Study staff will be trained by the PIs and their staff. Participants will be recruited by trained staff. Staff will conduct interviews in-person and by telephone. Study visits and clinical procedures will be performed according to the institutional standards. In-person visits may be conducted on campus at the CRC, at the OSU hospital, or at the Center for Tobacco Research. Bronchoscopy procedures may be conducted at the OSU main hospital pulmonary suite or the CRC by a board certified pulmonologist. PFTs may be performed by trained personnel such as those in the hospital's pulmonary department or CRC. Specimens will be collected by trained staff using Universal Precautions for Prevention of Transmitting Bloodborne Pathogens. Sample processing and laboratory analyses will be performed in the Shields' lab, the department of Pathology, OSUCCC shared resources, or outside laboratories. Study data will be analyzed by Shields' Lab staff.

Viral exposure precautions: As previously detailed, will follow institutional standards and best practices. Given the situation continues to evolve, study procedures may also adapt to address subject and personnel safety as well as study and institutional resources. Examples for assessing signs and symptoms related to COVID-19 or other viral illness are provided, but these practices may vary throughout the study as community and institutional conditions change.

Prior to all in-person visits, study staff will contact subjects to ask about possible COVID-19 symptoms. Additional precautions to mitigate exposure may include limiting physical interactions, when possible (e.g., remote/telephone visits or physical in-person distance), assessing signs and symptoms of viral illness (e.g., temperature and exposure assessments), and providing appropriate personal protective equipment (PPE) to subjects and staff (e.g., masks, gloves, googles, face shields, etc.). Surfaces, equipment, and materials will be sanitized prior to and after use. Staff and subjects will wash or sanitize their hands as warranted.

If institutional standards require COVID-19 testing, subjects may be asked to undergo testing prior to the bronchoscopy procedure. Testing procedures may be coordinated by staff and subjects may be asked to follow standard testing protocols. If a subject tests positive before the first bronchoscopy, the subject would be placed on a wait list for at least three months and re-tested before his/her appointment; if a subject tests positive before a follow-up bronchoscopy, his/her participation will be withdrawn by the PI.

Phone screening: Subjects interested in participating will be provided a phone number to call. Subjects recruited through the Information Warehouse/MD "opt out" approach will be contacted directly by study staff. The study will be briefly described using the phone guide. Subjects who are interested will be screened for eligibility

and exclusion criteria, and additional details will be provided. If subjects agree, they will be invited to attend a baseline/orientation session.

Baseline/orientation Visit: After phone screening and description of the study, subjects will be invited to participate in the study and attend an orientation visit where the eligibility criteria will again be confirmed, a medical history, medications, herbs, supplements and vitamins will be taken for review by Drs. Shields and the pulmonologist, and the bronchoscopy and preparations for the procedure will be explained. Following informed consent, subjects will complete a series of questionnaires (see below) to identify if the participant meets the eligibility criteria and baseline measures will be obtained. Following completion of the questionnaires, subjects' heart rate, blood pressure, oxygen level, height and weight will be recorded. Exhaled CO (Carbon monoxide) level will be measured. Potentially eligible subjects will be provided CoVita/Bedfont iCO Smokerlyzer® (https://www.icoquit.com/us/) for home CO daily monitoring and reporting real-time via the internet using a smart phone or tablet. The device measures CO ranging 0-100ppm and has sensor sensitivity of 1ppm with repeatability ≤±5%. This monitor features an easy to use App for both iOS and Android devices that will send the subject reminders to take a breath test as well as send results to the study team. To ensure the subjects are measuring their own CO, staff may also arrange for periodic remote visits by Zoom or another approved electronic platform to visually observe the testing and results. This device is frequently used for assessing CO in trials and observational studies. The pulmonologist and/or PI will review patient data to confirm eligibility. Subjects will then be scheduled for their bronchoscopy visit, ideally within two weeks. Subjects will be informed if there are any of their regular medicines, herbs, supplements or vitamins they should or should not take before their bronchoscopy. Subjects will be given 24 hour urine collection containers and instructions. Subjects will receive a reminder to begin 24-hour urine collection the day before their bronchoscopy.

Bronchoscopy Visit: At the first bronchoscopy visit, subjects will be randomized to examination of the right or left lung for their first bronchoscopy; the opposite lung will be assessed for the second bronchoscopy, to reduce the possibility that the assays in the 2nd bronchoscopy would be affected by the baseline one. The pulmonologist performing the bronchoscopy will obtain the medical history and diet examination to assess the potential risks of a bronchoscopy. Subjects with respiratory, cardiac, oral or other diseases that would place them at additional risk for bronchoscopy will be excluded from the bronchoscopy and the study. A urine pregnancy test will be conducted for women of childbearing potential (i.e., premenopausal and not surgically sterilized). Pregnant women will be excluded from the study. The procedure and risks will be explained. Pulmonary function will be measured by incentive spirometry. Subjects' heart rate, blood pressure, oxygen level, height and weight will be recorded. FeNO will be measured with the NIOX VERO® (http://www.niox.com/en-US/about-niox-vero/). Additional samples of saliva, 24-hour urine, nasal brushing and lavages, and blood will be collected for planned laboratory analyses. Approximately 2 ml of saliva will be collected by trained staff to examine salivary microbiome. An additional (parafilm induced if necessary) saliva sample, approximately 3 ml, will be collected to examine salivary biomarkers for tobacco use. The urine collection will be examined for osmolality and a variety of biomarkers. A spot urine sample of approximately 50mL may be collected immediately prior to the procedure for pregnancy tests or if there are concerns with the 24-hour collection. Nasal brushing and lavages will be collected by trained staff to examine for assessing non-invasive methods that might be surrogate markers for lung brushing and lavages. These specimens will be collected when the patient arrives and before the bronchoscopy is performed or any medications are administered. Approximately 15 mL of blood, for plasma and serum analysis, will be collected by the nurse. A bronchoscopy and brushing will be performed consistent with OSU approved protocol 2013H0142, 2015C0088 and current medical standards of care for our Division of Pulmonary Medicine in the Department of Internal Medicine.

Bronchoscopy and brushing procedure: Standard of care protocols for bronchoscopy at OSUMC will be utilized. The subject will be instructed to not eat or drink anything after midnight prior to the procedure (minimum 8 hours nothing by mouth prior to bronchoscopy). The patient will be instructed by study staff if they should take medications with a sip of water on the morning of the procedure. Bronchoscopy involves passage of a long, narrow, flexible tube through the nose or mouth, and then into the airways of the lung. If the volunteer agrees to participate, prior to passage of the tube, the nose, throat, and the area around the vocal cords are all locally anesthetized using Lidocaine or Cetacaine. Versed and/or Fentanyl is administered by intravenous catheter

placed in an arm or hand. Versed is a short acting benzodiazepine and Fentanyl is an opioid analgesic that will relax the subject. It is not absolutely necessary that the subjects receive sedation, and it can be the subject's choice to refuse Versed and/or Fentanyl if they wish. Sedation will be used unless the subject asks for it not to be given. If there is over-sedation with standard doses of Versed, Flumazinol can be used for immediate reversal of sedation. Monitoring of heart activity (EKG, electrocardiogram), pulse oximetry, and breathing is performed continuously throughout the procedure. In the case of an emergency, pulmonary and/or critical care board-certified physicians trained in advanced care for critically ill patients are immediately available and can perform life-saving care if needed, as would be typical for patients in the hospital. During this procedure, the physician performing the bronchoscopy will examine the lungs and place the scope in the specified lung segment. Lidocaine 1% is injected through the bronchoscope, usually 1 cc, as needed during the procedure to control the cough reflex. When the bronchoscope has been placed into a specific airway of either the right or left lung as identified by the randomization process, sterile salt water (about 3 ~ tablespoons) is placed into the lung and immediately suctioned back, washing off cells and materials lining the area. Fluid will be injected approximately 5-7 times recovering samples at each lavage. This will yield approximately 40-60 ml of bronchoalveolar lavage fluid. Next, the bronchoscopist will insert a cytology brush into bronchoscope and sequentially obtain approximately 4 to 5 brushings of grossly normal airway epithelium from the main bronchus of the lung. After each brush of epithelium, the cytology brush should be removed from bronchoscope and vigorously agitated into round-bottom tube labeled "BEC" containing 3 ml of RNAprotect cell reagent while the clinical coordinator or bronchoscopy assistant holds the tube. After depositing cells from last brushing into the tube, the clinical coordinator caps the tube and places on wet ice. Lastly, the bronchoscopist will smear the cytology brush onto a slide, turning the brush to ensure contact between the slide and each side of the brush. The brush may be retained.

The bronchoscopy typically takes 15 minutes and the entire procedure 30-60 minutes (several factors influence duration). When the procedure is finished, the subject's vital signs will be monitored until they return to baseline and the IV will be removed. The subject will be asked not to eat or drink anything for 2 hours after the procedure because their throat muscles will still be numb. The nurse or clinical staff will review discharge instructions. For procedures performed at the CRC, subjects who do not have a ride home from the procedure may stay at the CRC under supervision of CRC staff for 24 hours. This extended stay must be prearranged and will be paid for by the study. A chest x-ray may be required on rare occasions. All subjects will be called within 72 hours post-bronchoscopy to monitor symptoms.

Saliva collection and processing: Saliva will be collected when the participant arrives for the bronchoscopy visit. Subjects should abstain from brushing teeth, chewing gum, eating, or drinking for at least 1½ hours before the visit. Subjects will be asked the last time they ate, used mouthwash, and brushed their teeth. Subjects will be provided with a small (Dixie-cup sized) cup of tap water and asked to rinse their mouth vigorously for 30 seconds. After rinsing, the subject should expectorate the water into the cup or a sink. After expectorating the water, the subject should wait for two minutes before starting the saliva collection process. During the collection period, the subject should be seated upright with head tilted slightly forward. The subject should hold the styrofoam cup with the salivary tube inside on wet ice during the entire collection process. The funnel may be inserted into the salivary tube to assist with saliva collection. The subject will be instructed to minimize orofacial movements to decrease their influence on salivary flow. The subject should neither swallow nor speak during the entire collection process. The subject will be instructed to allow the saliva to accumulate in the floor of the mouth for 60 seconds without swallowing. After this period the subject should empty the entire accumulated saliva into the salivary tube on ice. The procedure will be repeated two more times for a total collection time of 3 minutes. The subjects will be instructed not to swallow during the entire collection period. At the end of the collection period the subjects will expectorate remaining saliva in their mouth into the test tube. The tube containing saliva is stored on wet ice, and transferred to the laboratory for processing and storage. An additional (parafilm induced if necessary) saliva sample, approximately 3 ml, will be collected. Subjects who have difficulty providing the quantity of saliva required will be instructed to chew a plastic (parafilm) wrap. Subjects will be instructed to not swallow the plastic wrap and to not spit the plastic wrap in the test tube. Subjects will be instructed not to swallow during the entire collection period. At the end of the collection period the subjects will expectorate remaining saliva in their mouth into the test tube. The tube containing saliva is stored on wet ice, and transferred to the laboratory for processing and storage. Processing should occur as soon as possible, not more than after one

hour of specimen collection. The specimen will be aliquoted into two 1 ml samples. If additional sample remains, additional tubes with labels will be used. Samples will be stored at -80.

Urine collection and processing: 24 hour urine containers will be provided to the subject. Females will be given "hats" for collection. Subjects will be verbally instructed on collection of the second morning void through the first morning void of the next day. Written instructions will be included in a bag with the urine supplies. A spot urine (~50mL spot) will be collected from the subjects at the bronchoscopy visits before any medications are provided and the procedure is performed. The urine will be aliquoted for laboratory analyses.

Blood collection and processing: Two blood tubes that allow plasma and serum analysis will be collected by the nurse. Two additional tubes of blood will be collected at the second bronchoscopy visit for nicotine boost assessment for subjects randomized to use the nicotine-SREC. Standard processing guidelines will be followed.

Nasal lavage fluids: Nasal lavages will be collected before any medications are provided for the bronchoscopy procedure. Based on prior studies [273, 274], and as we are doing now for OSU protocol 2015C0088, we will have subjects stand up with their heads anteverted at 45 degrees and ask him/her to breathe through their mouths to close the nasopharynx. Then, a syringe filled with 10 ml of saline (0.9% NaCl at room temperature) will be gently inserted into one nasal cavity. The saline will be slowly passed into the nasal cavity and back into the syringe up to five times. The same procedure will be repeated on the opposite nasal cavity using a new syringe. The total time for the irrigation will be approximately 5 minutes. The volume of the lavage fluid recovered after the nasal lavages will be recorded from each side.

Nasal brushing: Nasal epithelial cells will be collected [275, 276]. Briefly, brushing will be performed using a cytology brush (e.g., Cytology Brush by MP Corporation) after nasal lavage fluid collection. The brush will be inserted into nares and gently rotated (3-5 times) to brush the turbinate and then immediately placed in the collection tube containing a RNAprotect preservative reagent. Then, the brush will be shaken vigorously within the tube, so that the cells become detached from the brush.

Randomization: At the baseline bronchoscopy visit, subjects will be randomized before the procedure to one of the 4 groups (control and interventions). Those assigned to receive an intervention, will receive the product for free. All intervention groups will receive motivational counseling for smoking cessation. Study staff will be blinded regarding study groups. Medical personnel will be informed if she/he deems it pertinent or if the subject is assigned to receive the placebo and varenicline.

Standardized Research Electronic Cigarette: This study will utilize the NIDA SREC, which was developed under contract to NJOY (https://www.drugabuse.gov/funding/supplemental-information-nida-e-cig) so that it can receive Investigational Tobacco Product (ITP) designation for this study (https://www.fda.gov/TobaccoProducts/Labeling/RulesRegulationsGuidance/ucm463951.htm). The purpose of the ITP is for the FDA to allow for the study of tobacco products without pre-market evaluations that are required for medications and devices. Under current federal law, tobacco products that are not already on the market may be permitted by the FDA for research studies. With this application, NJOY will submit a master file that includes premarket data that the FDA will assess prior to determining the ITP status. Concurrent with this protocol, an ITP application also has been submitted. This application has already been reviewed and approved by NIDA. Without an ITP, NIDA will not allow the SREC product to be provided for use.

The FDA process to assess a product as an ITP, the FDA generally considers whether there are controls on how and to whom the tobacco products intended for investigational use are distributed; whether the protocol for the clinical investigation or the procedures used during the clinical investigation adequately provide for the protection of human subjects; whether the study is designed to ensure the quality and integrity of the study data and permit other investigators to replicate the findings; and. whether there are adequate procedures in place to ensure that investigational tobacco products are not commercialized.

NJOY (https://www.njoy.com/mission) markets itself as ". . . a pioneer in the electronic nicotine delivery system (ENDS) market, and is today the largest independent e-cigarette and vaping company in the USA. NJOY is the only major vaping company to offer products across all form factors; disposable and rechargeable "e-cigalikes", "open system" e-liquids and vaping devices, and advanced "closed system" e-liquids, and across all major channels of distribution; convenience stores, drug stores, mass merchandisers, vape shops, and on-line. . . NJOY's mission is to end smoking-related death and disease by offering preferred alternatives to adult smokers and vapers around the world. In doing so, they hope to make the combustion cigarette obsolete."

The SREC was developed by NJOY and is based on a currently marketed product, the NJOY Ace. The device and sealed pod are shown below.

Device specifications:

Dimensions - 74.12 mm (L) x 29.85 mm (W) x 13.50 mm
Weight - Device 54g ± 5g, filled Pod 7.5 ± 0.5
Device is packaged with USB cable
Pod characteristics – sealed and disposable
Puffs per pod - anticipated number of standardized, 3-second, 55mL puffs that correspond to the usable pod lifecycle (i.e. No Visible Liquid (NVL) in pod) is 300 puffs. Study results demonstrate a mean puff count of 299 puffs to NVL, with a minimum observed puff count of 280 and maximum observed puff count of 330
Volume of liquid per pod 1.9 mL
Rechargeable battery characteristics - Number puffs (3s) per charge ~200
>80% initial capacity) - 300 charge cycles
Time to full charge via line power - 75 – 85 min
Output voltage - 2.2-3.1 V
Battery Storage Capacity >/= 400 mAh

E-liquid Characteristics:

Nicotine-containing and placebo versions are "tobacco" flavored
Shelf-life at 25C - > 1 year (unopened)
Nicotine concentration - 5% w/w (corresponds to 58 mg / mL)
Propylene Glycol % - ~ 38.75% (wt / wt)
Glycerin % - ~ 50% (wt / wt)

Product use: At the first bronchoscopy visit, SREC users will be trained to use the product and given instructions on how to use the device. They will not be requested to use the device at the visit, but to use it at home representing the way real world e-cig users learn to use the product (product use will be observed one week later). An instructional video showing how to use the device may be provided at first bronchoscopy visit, and other visits as needed (https://www.youtube.com/watch?v=lKcblA_AbTg). Similar videos will be created for the other products as needed. Subjects in the SREC arms will be instructed that after two weeks of use, as they

learn to use the product and work towards complete substitution, they will begin their assignment effective on day 15.

E-cig users will be encouraged to use the product every 1-2 hours, and as needed to address cravings. Subjects will be allowed to smoke their usual brand of cigarettes *ad libitum* for the first 2 weeks and encouraged to reduce their cigarettes per day to 5 or less, followed by quitting cigarette smoking and avoiding cannabis use. Subjects will be contacted by phone or email after 72 hours of study product use, and there will be contact with the subject on a weekly basis (phone or visit). This two-week run-in period allows the subject to become accustomed to the product and have a specific quit date.

Subjects randomized to the placebo SREC will be offered varenicline, an FDA-approved smoking cessation medication, at no cost to limit withdrawal symptoms and increase study retention. Those participants will be given the medication at the day 8 visit, one week before the day 15 quit date.

Subjects randomized to NRT complete substitution will be instructed how to use the assigned product(s). Subjects will be asked to use patches (21mg) in combination with gum (4 mg) and/or lozenge (4 mg) . If appropriate, subjects may use a single product after discussion with study staff and/or the study PI. If subjects experience side effects, dosages can be reduced and/or products changed. The NRT group will begin to use the NRT at the day 8 visit, one week before the day 15 quit date.

All intervention subjects will receive counseling with motivational interviewing in order to achieve compliance.

SREC Dispensing (targeted, but may be revised as needed): Participants will be provided two devices, in case one fails, including charging materials. Based on email and telephone conversations with the SREC manufacturer (NJOY), NIDA, and Dr. Mark Eisenburg, a Cardiologist and Investigator at McGill University who is currently conducting a randomized clinical trial (Canadian E3 Trial) using a SREC device, we will assume that participants will use one tank/pod every two days (i.e., 0.5 tank/pod per day). At the anticipated rate, there will be 70 days on product or approximately 35 pods required.

Pods will be weighed and logged prior to dispensing. At each visit, the participant will be provided with a sufficient quantity of product for anticipated use and an additional 1 week of product. This will allow the participant to have enough product if the appointment is postponed or the participant uses more than anticipated. At the next visit, the participant will return used and unused product. Returned materials will be weighed. Unused/unopened product will be re-dispensed along with the necessary amount of new product. These estimates will vary depending on individual use. Dispensing will be adjusted to match product use. Participants can contact study staff to request additional product as needed. Participants will be asked to return all unused product and the devices at the end of the study.

Day	**Days between visits 7**	**Pods to be dispensed***
Day 1 (W0) – BR1	7	7
Day 8 (W1) – visit	7	7
Day 15 (W2) – visit	7	7
Day 22 (W3) – visit	7	7
Day 29 (W4) – visit	14	11
Day 43 (W6) – visit	14	11
Day 57 (W8) – visit	14	11
Day 71 (W10) – visit	NA	

*One week of additional product (~3 or 4 pods) may be distributed at each visit to provide sufficient quantity for any unanticipated use or delay in visit scheduling.

Varenicline dosing and dispensing (targeted, but may be revised as needed): Subjects randomly assigned to the Complete Substitution with the placebo SREC device will be offered up to 12 weeks of varenicline, a nicotinic receptor partial agonist indicated for use as an aid to smoking cessation treatment at no cost. The 12 week

duration extends the use up to one month after the intervention to be consistent with FDA-approved uses. Dr. Wewers (or other MD associated with the study) will review all subjects' medical history and evaluate the subject for indications and safety of the varenicline at the BR1 visit prior to the procedure. Subjects may continue in the study if they are not eligible to use varenicline or refuses.

The medication (see below) will be obtained from the OSUWMC or James Cancer Center pharmacy in bulk. Medications will be stored at room temperature (as recommended by the manufacturer) and in locked cabinets.

Packs
Starting 4-week card: 0.5 mg × 11 tablets and 1 mg × 42 tablets NDC 00069-0471-03
Continuing 4-week card: 1 mg × 56 tablets NDC 00069-0469-03
Starting Month Box: 0.5 mg × 11 tablets and 1 mg × 42 tablets NDC 00069-0471-03
Continuing Month Box: 1 mg × 56 tablets NDC 00069-0469-03

Bottles
0.5 mg - bottle of 56 NDC 00069-0468-56
1 mg - bottle of 56 NDC 00069-0469-56

Medication will be labeled with the subject's study ID, "For Research Purposes", Study telephone number, Medication Name and strength, Instructions for use, Dispensed date, Expiration date, and Quantity. The research staff dispensing the medication will include their initials on the label and document the dispensation (and return of unused medication) on the product accountability log. Upon completion of the study or if the subject stops taking the medication, any medication that is returned by the subject will be taken to the pharmacy for appropriate disposal.

A general accounting of all varenicline medication obtained from the pharmacy and dispensed to subjects will be maintained by study personnel.

At each visit, the participant will be provided with a sufficient quantity of product for anticipated use and an additional 1 week of medication or given a 4 week card. This will allow the participant to have enough medication if the appointment is postponed. At the next visit, the participant will return unused medication, which will be recorded and redispensed as appropriate. Subjects will be monitored for adverse reaction using the same process as all other arms.

Patients will begin varenicline dosing one week before the day 15 quit date.
Starting Week: 0.5 mg once daily on days 1–3 and 0.5 mg twice daily on days 4–7.
Continuing Weeks: 1 mg twice daily for up to a total of 12 weeks.

Day	Days between visits+7	Daily dose	Tablets to be dispensed
Day 8 (W1) – visit	7 + 7 = 14	0.5 mg once daily for 3 days, then 0.5 mg twice daily for 4 days	22 tablets of 0.5 mg
Day 15 (W2) – visit	7 + 7 = 14	1 mg twice daily	28 tablets of 1 mg
Day 22 (W3) – visit	7 + 7 = 14	1 mg twice daily	28 tablets of 1 mg
Day 29 (W4) – visit	14 + 7 = 21	1 mg twice daily	42 tablets of 1 mg
Day 43 (W6) - visit	14 + 7 = 21	1 mg twice daily	42 tablets of 1 mg
Day 57 (W8) – visit	14 + 7 = 21	1 mg twice daily	42 tablets of 1 mg
Day 71 (W10) – BR2			Up to three weeks (21 tablets) to meet the FDA recommended duration

NRT dosing and dispensing (targeted, but may be revised as needed): Similar to the other conditions, NRT will be dispensed at each visit. Beginning at Day 8 (W1), the participant will be provided with a sufficient quantity of product for anticipated use and an additional 1 week of product. This will allow the participant to have enough

NRT if the appointment is postponed. At the next visit, the participant will return unused NRT, which will be recorded and redispensed as appropriate. Subjects will be monitored for adverse reaction using the same process as all other arms. The NRT will be distributed assuming the maximum allowed per day is 1 patch, 20 lozenges, and 24 pieces of gum.

Day 8/ Wk1 visit: One week after the baseline bronchoscopy, subjects will return (day 8) to report their tobacco and/or product use status, and to report any health issue. Subjects will be offered the opportunity to complete the questionnaires online and measure their breath CO within 24 hours before their scheduled visit. Subjects will complete questionnaires, and CO will be assessed. Blood pressure, heart rate, oxygen level and weight will be recorded.

Subjects in the SREC arms will be reminded that after one week of use as they learn to use the product and work towards complete substitution, they will begin their assignment effective on day 15. SREC users will be encouraged to use the product as frequently as needed to address cravings, allowed to smoke their usual brand of cigarettes *ad libitum* for another week, and encouraged to reduce their cigarettes per day to 5 or less, followed by quitting cigarette smoking and avoiding cannabis use. Subjects randomly assigned to the Complete Substitution with the placebo SREC device will be provided with appropriate dose/amount of varenicline and will be instructed to use it as indicated above.

Subjects randomized to NRT complete substitution will be provided patches in combination with gum and/or lozenge unless otherwise discussed, and instructed how to use these products. Dosing will be determined based on their baseline consumption and time to first cigarette. Subjects will be allowed to use a combination of products (e.g., patch and gum/lozenge) in order to achieve cessation. If subjects experience side effects, dosages can be reduced.

All subjects randomized to receive an intervention will receive counseling with motivational interviewing in order to achieve compliance.

Subjects who indicate difficulty maintaining a reliable source of communication (i.e., telephone, internet access) after enrolling in the study may be provided a cell phone to facilitate communication, data collection via internet, and improve retention. The cell phone and a data plan may be provided at no cost to the participant. Subjects will be instructed to return the phone at the end of the study. Phones will be erased and reused as needed. No data from the phone will be captured or retained.

Day 15/ Wk 2, Day 22/ Wk 3, Day 29/ Wk4, Day 43/ Wk6 and Day 57/ Wk 8 visits (+/- 3 days): Subjects in the intervention arms will begin their assignment (stop smoking) effective on day 15. Subjects will be offered the opportunity to complete the questionnaires online and measure their breath CO within 24 hours before each scheduled visit. At each visit, blood pressure, heart rate, oxygen level and weight will be recorded. Subjects will complete questionnaires. CO will be assessed. Subjects in the intervention groups will be asked to bring back their used and unused product and will be provided additional pods, varenicline and NRT as appropriate. Subjects who fail to return product or report daily use may be identified as non-compliant and removed from the study by the PI on a case by case basis. At day 57/Wk 8 visit, subjects will be given 24 hour urine collection containers and instructions. Subjects will receive a reminder to begin 24-hour urine collection the day before their bronchoscopy.

Follow-up bronchoscopy: At 10-weeks (8 weeks on the experimental condition and a 2 weeks run-in period), subjects randomized to the SREC groups will be instructed to use the assigned device immediately prior to the visit. During the visit, these subjects will demonstrate use and inhalation will be assessed in a closed room with negative pressure or a “smoke eater” device that will reduce the exposure to exhaled particles. Windows or webcams may be utilized to observe and communicate with participants during this exercise. No webcam images or audio data will be recorded or retained without the participants’ permission.

Inhalation of the SREC will be assessed by the Great Lakes Neurotechnology BioRadio System, which includes

2 respiration inductance plethysmography bands (thoracic and abdominal) and the VivoSense Advanced Respiratory Analysis Module and VivoSense Core Physiological Analysis Software. The subject's inhalation data will be recorded for approximately 2 minutes/15 breaths using a spirometer, then approximately 1 minute without the spirometer, before e-cig use, then while using e-cig for 10 puffs over 4.5 minutes, and lastly for an additional 1 minute after using the e-cig. A nicotine boost will be concurrently assessed for nicotine-SREC users (See below).

All subjects will undergo an additional bronchoscopy (the same procedure and measures as above). Subjects will be reminded that e-cigs are still a new product, and much is still unknown about the potential benefits of e-cigs for fostering smoking cessation, and the numerous types of harm that they could cause, both on an individual level and a population basis, and they will be advised not to use black market devices or modify commercial devices. Study staff will emphasize the importance of being e-cig and tobacco-free. If needed, subjects will be offered cessation services by referral to the national quit line (1-800-QUIT-NOW). All subjects will be called within 72 hours post-bronchoscopy to monitor symptoms. All subjects will be called 3 months after study completion to assess their tobacco use, including e-cig use. Subjects are asked to return SREC devices and all unused product.

Scheduling visits: All visits and calls will have a small window (+/- 3 days) in which study procedures can be conducted without being recorded as a deviation.

Compensation and Retention/Compliance Bonus: Compensation will be provided weekly and/or at in-person visits using a reloadable ClinCard or similar method. Compensation listed below includes travel. If a subject reports transportation barriers for non-bronchoscopy visits, study staff will attempt to arrange alternative transportation (e.g., Lyft). Participants may have up to $10 deducted from their visit compensation if the service is utilized and paid for by the study. Compensation for procedures and travel was determined by using existing studies (e.g., 2003H0142, 2015C0088, 2019C0082, 2020C0169) and consulting with several practitioners in the Pulmonary Department, as well as the DSMB.

Visit		**Compensation**
Orientation	Visit	$60
Day 1 (W0) Bronchoscopy 1	Visit / procedure	$250
Day 4	Call	$5
Day 8 (W1)	Visit	$40
Day 15 (W2)	Visit	$40
Day 22 (W3)	Visit	$40
Day 29 (W4)	Visit	$40
Day 36 (W5)	Call	$5
Day 43 (W6)	Visit	$40
Day 50 (W7)	Call	$5
Day 57 (W8)	Visit	$40
Day 64 (W9)	Call	$5
Day 71 (W10) Bronchoscopy 2	Visit / procedure	$300
3 month follow-up	Call	$10
RDS (Recruitment Referrals)		Up to $60
Bonus (Daily CO, Random Visit)		Up to $200
Total		**Up to $1140**

Bonus: Subjects' at home CO daily monitoring (via iCO) and reporting will be incorporated into a contingency management bonus. Using a threshold of 6 ppm, subjects with CO levels ≤6 ppm ≥5 days per week will receive an additional $20 per week ($160 total). To address compensation equity among the conditions, subjects assigned to the usual brand smoking arm will be compensated if they send CO results ≥5 days per week.

Subjects will be eligible to receive a $40 bonus at the end of the study if the BR2 urine sample confirms the subject followed study instructions for using the product, medication, or device as assigned **and** if all aspects of a randomly selected visit were completed (e.g., surveys, returned product and/or packaging, etc.). If the subject does not provide a 24 hour urine sample at BR2 or laboratory analysis indicates the subject did not follow study instructions, the bonus will not be provided. If the randomly selected visit is missed or instructions were not followed, it will be considered as "not following instructions" and the subject will not receive the bonus. The bonus will be given after the 3 month follow-up call or as appropriate (e.g., upon withdrawal).

RDS: Subjects may choose to invite individuals they believe to be smokers, age 21 years or older and who are willing to be screened to join the study. Subjects can earn $10 for each person who meets those criteria and is phone screened. They can earn up to $60 total. The RDS compensation was informed by another study using this approach (2017H0439) and aligns with the time and effort required.

If a subject is found to be ineligible at time of the procedure, the subject will be given $25 for their time, plus $7 for transportation or parking.

Contacting subjects: Subjects will be contacted up to 10 times over a 2 week window for participation, but no more than 3 voicemails requesting response will be left. Thus, if there is no option for voicemails, there will be up to 10 attempts during the day or evening. If unable to reach the participant, a letter will be sent asking for the participant to call us.

ii. Timeline

Timetable for completion of study aims: Month	**1-2**	**2-18**	**18-24**
IRB approval, train staff, advertisements, begin enrollment	X		
Accrual		X	
Follow-up procedures		X	X
Analyze data, manuscript preparations			X

d. Data Collection and Management Process

i. Data to be collected

The schedule of measures is provided in Table 1.

Baseline/orientation visit: At the baseline visit, ideally less than two weeks before bronchoscopy, subjects will complete questionnaires, as indicated in Table 1. Exhaled CO , blood pressure, heart rate, oxygen level and height and weight will be assessed.

Bronchoscopy visits: At bronchoscopy visits, subjects will complete questionnaires, as indicated in Table 1. Blood pressure, heart rate, oxygen level and weight will be assessed. CO, FeNO and PFTs will be measured. General changes in diet and physical activity over the course of the study will be assessed at the second bronchoscopy visit. Product evaluation will be administered at the follow-up bronchoscopy to assess the experience with the SREC or NRT. Inhalation will be assessed at the follow-up bronchoscopy for subjects assigned to one of the e-cig use conditions, and a nicotine boost will be concurrently assessed for nicotine-SREC users. At the second bronchoscopy visit, the same measures will be provided. These measures are currently used in other studies, including a study involving bronchoscopy.

Day 8/ Wk1, Day 15/ Wk 2, Day 22/ Wk 3, Day 29/ Wk4, Day 43/ Wk6 and Day 57/ Wk 8 visits: At each in-person visit, subjects will complete questionnaires, as indicated in Table 1. Blood pressure, heart rate, oxygen level and weight will be assessed. CO will be measured. The product compliance, product accountability log and product evaluation scale will be used to assess the experience with the SREC or NRT.

Phone calls: During the weeks when there are no visits, subjects will be called on the phone to assess tobacco and/or product use, toxicity and health status. Subjects assigned to one of the intervention groups who need additional abstinence counseling beyond the planned visits and calls will be instructed to contact study staff. All subjects will be called within 72 hours post bronchoscopy to monitor symptoms and at 3 months after study completion to assess CESD, Expectancies, Drug Effects/Liking, stages of changes, FTND or modified FTND, as appropriate, product evaluation scale and their tobacco use, including e-cig use.

Daily tobacco use: Subjects will be asked to report their daily tobacco and/or product use by phone or internet.

Table 1. Data Collection

Measures	ORNT	Day 1/ BR1	Day 8/ Wk1	Calls 1-4: Day 4 Day 36 Day 50 Day 64	Day 15/ Wk2 Day 22/ Wk 3 Day 29/ Wk4 Day 43/ Wk 6 Day 57/ Wk8	Day 71/ BR2	3 Months Post BR2 Call
Contact & Physician Information	x						
Demographics	x						
Progress Note	x	x	x	x	x	x	x
Medical History (Baseline and Updates)	x	x	x	x	x	x	
Covid-19 Screener	x						
Covid-19 Follow Up	x	x	x	x	x		
Adverse Events	x	x	x	x	x	x	x
Concomitant Medications	x	x	x	x	x	x	x
Prime MD	x						
CESD	x				**Day 29**	x	x
SMAST	x						
Perceived Stress		x			**Day 29**	x	
Perceived Health Risk		x			**Day 15**	x	
Tobacco Use History*	x						
Social/Lifestyle History	x					x	
Environmental Exposure	x	x			**Day 29**	x	
Health Changes (Overall Health)**	x	x	x	x	x	x	
Alcohol and Drug Abuse History	x						
Alcohol Use***	x						
Supplements Use	x						
Occupational Exposure	x	x			**Day 29**	x	
Eligibility Checklist	x						
Smoking Dependence Motives (WISDM)	x		x		x	x	
Stages Of Change	x					x	x
FTND	x	x	x		x	x	x
FTND (Modified for SREC Users)					x (e-Cig)	x (e-Cig)	x (e-Cig)
MN Withdrawal Scale		x	x		x	x	
Smoking Urges		x	x		x	x	
Product Evaluation Scale (NRT)					x	x	x
Evaluation of Electronic Vaping Devices			x	x	x	x	x
Expectancies			x (e-Cig)		x (e-Cig)	x (e-Cig)	x (e-Cig)
Drug Effects/Liking			x (e-Cig)		x (e-Cig)	x (e-Cig)	x (e-Cig)

Interest In Switching or Quitting	x					x	
Vitals (Including Height and Weight)	x	x	x		x	x	
CO (Daily)	x	x	x	x	x	x	
FeNO Results		x				x	
24-Hour Urine Sample Collection Form		x				x	
Inhalation Assessment						x (e-Cig)	
Pulmonary Function Test		x				x	
Sample Collection Form		x				x	
Pre Bronchoscopy Questionnaire		x				x	
Lung Randomization		x				x	
BAL Collection Information		x				x	
Product Accountability Log		X (e-Cig)	x		x	x	
E-cig Cartridge/ Pod Weight Log		x (e-Cig)	x (e-Cig)		x (e-Cig)	x (e-Cig)	
Daily Use Summary (TLFB)	x	x	x	x	x	x	x
Daily Diary		x	x	x	x	x	
Product Compliance****		x (e-Cig)	x	x	x	x	
Cessation Counseling*****			x	x	x	x	x
Follow-Up Call							x
Change In Status	As Needed						
Protocol Deviation Report	As Needed						

*** Subjects will be asked to report their daily tobacco and/or product use by phone or internet**
****Similar to Respiratory and Global Health Questionnaire**
*****Follow-up alcohol use is captured on the Daily Use Summary (TLFB)**
******Product Compliance will be administered to SREC subjects starting at BR1; NRT subjects will be asked to complete the questionnaire starting at Day 8/Wk 1**
*******Cessation counseling will occur at each visit/call after BR1 and/or as often as needed on an individual basis**

Table 2. Sample Collection

Sample	ORNT	Day 1/BR1	Day 8/ Wk1	Day 15/Wk 2 Day 22/Wk 3 Day 29/Wk 4 Day 43/Wk 6 Day 57/Wk 8	Day 71/BR2 Call
Saliva		x			x
Urine*		x			x
Urine (24 hour)		x			x
Blood		x			x
Nasal lavages and brushing		x			x
Bronchoalveolar lavage and brushing		x			x

* A spot urine sample may be collected immediately prior to the procedure for pregnancy tests or if there are concerns with the 24-hour collection.

Table 3. Planned analyses

Type of analysis	ORNT	Day 1/BR1	Day 8/ Wk1	Day 15/Wk 2 Day 22/Wk 3 Day 29/Wk 4 Day 43/Wk 6 Day 57/Wk 8	Day 71/BR2
Saliva					
RNA microbiome		x			x
Biorepository		x			x
Blood					
Cannabinoids		x			x
Glycerol and propylene glycol		x			x
Nicotine boost levels					x
Marijuana/THC		x			x
Biorepository		x			x
Urine					
Marijuana/THC		x			x
Pregnancy test (female only)		x			x
Cannabinoids		x			x
Tobacco use biomarkers*		x			x
Osmolality		x			x
Biorepository		x			x
Bronchoalveolar lavage					
Total and inflammatory cells count		x			x
Cellular component analysis		x			x
Inflammatory cytokines		x			x
Glycerol and propylene glycol		x			x
Untargeted metabolomics		x			x
RNA microbiome		x			x
CyTOF Analysis		x			x
Oil Red O stain		x			x
Biorepository		x			x
Bronchial and Nasal Brushing					
miRNAs expression		x			x
miRNAs gene expression		x			x
DNA methylation		x			x
mtDNA genetic variations		x			x
Proteomics analysis		x			x
Biorepository		x			x

***Tobacco use biomarkers including but not limited to: Creatinine, Cotinine, 3-hydroxycotinine, TNE, PAHs, Phe T, Anatabine and Anasabine, Nicotelline, Total NNAL, 2-HPMA, 3-HPMA, HMPMA, AAMA, CNEMA, HEMA, MHBMA-1+2, MHBMA3, MMA, S-PMA, 8-*iso*-$PGF_{2\alpha}$ (8-isoprostane), 11-dehydrothromboxane B2 (11-dTXB2), PGE-M and Catecholamines**

ii. How the data will be collected or accessed

Patient data will be collected using an electronic platform such as Qualtrics/REDCap or hardcopy through personal interview. IHIS may be used to facilitate lab processing with pathology. The bronchoscopy procedure and potential need for COVID-19 precautions may require accessing the participants electronic medical records. Results from bronchoscopy and COVID-19 test may be included in the participant's medical record. Only authorized study personnel will have access to the data.

iii. Laboratory Procedures

The following are planned procedures:

- Blood pressure, heart rate and oxygen level
- Physical examination
- Height and weight
- FeNO measurement
- CO measurement
- Pulmonary function tests
- Inhalation assessment

- Blood:

  (1) Glycerol and propylene glycol-- mass spectrometry methods. The assays will be developed and performed in the Nutrient Phytochemical Shared Resource of the OSUCCC, as previously described [182, 183, 290]. Cannabinoids will be measured by mass spectrometry methods [291]. Tetrahydrocannabinol (THC) is a well-established metabolite to confirm abstinence of the consumption of marijuana use.

  (2) Nicotine boost levels will also be obtained. Subjects will be instructed to use the device immediately prior to the visit. Time of last use will be recorded. Blood will be collected immediately prior to the use of the device. The subject will puff the e-cig using at least three puffs over 10 minutes, and a second blood draw will be done immediately after last puff.

- Urine:

  (1) Pregnancy test for women of childbearing potential (i.e., premenopausal and not surgically sterile).

  (2) Glycerol and propylene glycol-- mass spectrometry methods. The assays will be developed and performed in the Nutrient Phytochemical Shared Resource of the OSUCCC, as previously described [182, 183, 290].

  (3) Cannabinoids will be measured by mass spectrometry methods [291]. Tetrahydrocannabinol (THC) is a well-established metabolite to confirm abstinence of the consumption of marijuana use.

  (4) Tobacco use biomarkers: urine will be analyzed for validation of smoking cessation, the measurement of extent of tobacco use, and assessing exposure. This will include but not limited to: Creatinine; cotinine (metabolite of nicotine), nicotelline, 3-hydroxycotinine, total nicotine equivalents (TNE), polycyclic aromatic hydrocarbons (PAHs, Phe T), total NNAL (metabolite of tobacco-specific nitrosamines), anatabine and anasabine (tobacco alkaloids), mercapturic acids (metabolites of volatile organic compounds) including: 2-HPMA, 3-HPMA, HMPMA, AAMA, CNEMA, HEMA, MHBMA-1+2, MHBMA3, MMA, and S-PMA, 8-iso-PGF2α (8-isoprostane), 11-dehydrothromboxane B2 (11-dTXB2), PGE-M , Catecholamines, and untargeted metabolomics.

  (5) Osmolality.

- Bronchoalveolar lavage:

  (1) Total and inflammatory cells: Total cell counts will be measured by 1:1 dilution in trypan blue and instillation into disposable Countess™ cell counting chamber slides [292]. Automated cell counts and viability will be evaluated by the Countess® Automated Cell Counter according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). Differential counting will be performed on Diff-Quik stained cytospins [293] and at least 200 cells will be counted on each preparation according to standard morphologic criteria under light microscopy. Differential cell counts will be evaluated by a histopathologist at OSUCCC.

(2) Cellular component: Part of cells recovered from the BAL and nasal fluid will be lysed to measure expression of innate immune regulatory molecules. The remaining cells will be cultured up to 24h to determine their responsiveness to exogenous stimuli by quantitating newly released and newly produced intracellular molecules.
(3) Cytokines: Inflammatory cytokines, TNF alpha and total protein concentration in the lavage fluid will be assessed using laboratory techniques including but not limited to MSD and ELISA assays, and cellular expression of these will be assessed on the cellular components by immunohistochemistry as previously described [294].
(4) Glycerol and propylene glycol: Mass spectrometry methods. The assays will be developed and performed in the Nutrient Phytochemical Shared Resource of the OSUCCC, as previously described [182, 183, 290].
(5) Untargeted metabolomics: mass spectrometry methods. These assays will be done in the Nutrient Phytochemical Shared Resource of the OSUCCC.
(6) Cytometry by Time of Flight (CyTOF) analysis: Using a mass spectrometry technique based on inductively coupled plasma spectrometry and time of flight mass spectrometry will determine the cellular expression, properties of cells (cytometry) to distinguish inflammatory cell subtypes.
(7) Tobacco use biomarkers: will be analyzed for validation of smoking cessation, the measurement of extent of tobacco use, and assessing exposure. This will include cotinine (metabolite of nicotine), nicotelline, 3-hydroxycotinine, polycyclic aromatic hydrocarbons, NNAL (metabolite of tobacco-specific nitrosamines), anatabine and anasabine (tobacco alkaloids), mercapturic acids (metabolites of volatile organic compounds, and untargeted metabolomics.
(8) Acrolein DNA adducts: Total DNA will be extracted using commercially available kits (e.g., Qiagen AllPrep DNA/RNA Kit) and used to analyze acrolein DNA adducts as described previously [295].
(9) Oil Red O: Staining will be performed by the OSUCCC Pathology Department to examine cellular morphology.
(10) Lipidomics: mass spectrometry methods. These assays will be done in the Nutrient Phytochemical Shared Resource of the OSUCCC and at the Centers for Disease Control and Prevention (Atlanta, GA).

- Bronchial and nasal brushing:

(1) MicroRNAs and whole transcriptome: Total RNA containing small RNAs will be extracted from bronchial brushing specimens using commercially available kits (e.g., Qiagen AllPrep DNA/RNA Kit) and used for both gene expression using the Affymetrix GeneChip® Human Transcriptome Array and for miRNA expression using the Affymetrix GeneChip® miRNA Array, both available in the Genomics Shared Resource of the OSU Comprehensive Cancer Center and extensively used in the past by the Shields Lab [296, 297]. Expression will separately be assessed through RNAseq. Total RNA will be extracted from lavage cells and saliva for comprehensive profiling of microbiome using commercially available kits (e.g., QIAGEN miRNeasy kit). Sequencing will be performed in the Genomics Shared Resource of the OSU Comprehensive Cancer Center.
(2) DNA methylation: Total DNA will be extracted using commercially available kits (e.g., Qiagen AllPrep DNA/RNA Kit) and used for genome-wide DNA methylation profiling using Illumina Infinium Methylation EPIC BeadChip (Illumina) in the Genomics Shared Resources at Roswell Park Cancer Institute (Buffalo, NY).
(3) mtDNA genetic variations: Total DNA including mtDNA will be extracted using commercially available kits (e.g., Qiagen AllPrep DNA/RNA Kit) and used for mtDNA mutation using Hiseq Next Generation Sequencing (NGS) and for mtDNA contents using quantitative polymerase chain reaction (qPCR).
(4) Proteomics analyses: Proteins from epithelial cells will be digested into peptides and separated by reversed phase chromatography prior to infusion into a Q-Exactive-HF electrospray tandem mass spectrometer.
(5) Acrolein DNA adducts: Total DNA will be extracted using commercially available kits (e.g., Qiagen AllPrep DNA/RNA Kit) and used to analyze acrolein DNA adducts as described previously [295].

- Bronchoalveolar lavage cells and saliva for RNA microbiome:
  Samples will be analyzed to determine their bacterial composition using the TruSeq SBS Kit v3 Illumina Chemistry implemented on the HiSeq 2500 (Illumina, Inc.). This method can evaluate bacterial composition of the microbiome, including those not previously cultivated. RNA extraction, amplicon PCR, library preparation and sequencing will be carried out using microbiome optimized and sterile procedures, with microbial free certified reagents and consumables. Sequencing libraries QC will be assessed with Agilent BioAnalyzer HS DNA chip and Qubit Fluorometry analysis for library profile and amount, respectively. Libraries with compatible Illumina sequencing indices will be pooled together to be sequenced in an Illumina paired-end 50bp flow cell to at least 40 million pass filter reads/sample. The reads have adapters removed and trimming occurs by removing highly abundant reads such as human rRNA and tRNA using Bowtie2. Remaining reads are aligned using STAR to a database consisting of human genome version GRCh37.75 and bacterial and viral genomes databases hosted by NCBI to create an alignment file called a BAM file.

- Lipidomics analysis:
  Bronchoalveolar lavage fluid samples are extracted by liquid-liquid separation via Bligh and Dyer. Extracts are dried and reconstituted to an appropriate concentration in 1:1 dichloromethane/methanol containing ammonium acetate. Analytical samples are introduced via infusion through an electrospray probe to a Sciex Qtrap 5500 mass spectrometer (Sciex, Concord, Canada) enabled with Selexion differential ion mobility (DMS). Compound-specific DMS separations in positive or negative mode are followed by MRM transitions yielding >1,200 lipid features covering 13 different lipid classes. Semi-quantitative results are generated for comparative data analysis.

iv. Data collection timeline

The orientation/baseline visit including consenting process will take approximately 3 hours. The bronchoscopy visit will take approximately 2 hours (samples, physiologic measures, surveys), with 1-2 hour post-bronchoscopy observation period. Email contacts, calls and/or biweekly visits and procedures will take approximately 30-60 minutes. In total, participant time is estimated to be less than 20 hours.

v. How the data are stored and protected

A platform such as Qualtrics or REDCap or hard copy will be used to capture, store, and protect data. The first two platforms provide a secure, web-based application that is flexible enough to be used for a variety of types of research, provides an intuitive interface for users to enter data and has real time validation rules (with automated data type and range checks) at the time of entry. All data collected using the electronic data entry system will be stored on secure CCC servers. All data that are obtained and/or stored for this study will be maintained in confidence according to the rules and regulations of the Arthur G. James Cancer Hospital and the Richard J. Solove Research Institute, The Ohio State University, and HIPAA requirements.

Several measures will be in place to ensure the safety and confidentiality of data. First, the database will be maintained within the OSUMC firewall. All information that could identify participants, such as name, address, etc. will be maintained on this secured database with access limited to authorized study personnel. Second, the database requires user authentication in order to access and view the system. Currently, these electronic installations support electronic signatures by positively identifying the user through a unique username and password combination. For this study, only authorized study personnel will be given usernames and passwords. Computers used by staff will also be password protected, with network-based, inter-site communications of confidential information being encrypted. Third, these platforms are able to link participant data through the use of a unique study ID/number. Participants will be assigned a unique study ID, which will not contain any identifying information. The unique study IDs will then be used to identify participants throughout the study database. Fourth, these platforms can accommodate the creation and export of de-identified datasets that allows for the shifting of dates for the limited dataset export. Thus, the database platform will ensure the protection and confidentiality of any data that will be exported. Lastly, an ongoing computer virus protection program will be available and utilized, maintained, and audited on all computers and pathways.

Electronic and Paper Records. Data from questionnaires, physiologic measurements, and the various laboratory and data analyses will be stored similarly in password secured databases (under control of the data manager), and without protected health information. Any paper documents (e.g., informed consent forms) will be kept in a locked cabinet in a locked room inaccessible to staff other than authorized personnel.

We will maximize efforts to ensure confidentiality through the use of unique subject numbers. This number will be used on the participants' records. Data will be provided to the statisticians and/or analysts for analysis purposes. No identifying information will be provided; only the unique subject numbers will be included. Only limited project staff at OSU will have simultaneous knowledge of participant identities, subject numbers, and all related data for all participants. Limited project staff may include the following individuals: Program Director, Physicians, Consenter, and other bronchoscopy procedure staff. All staff will be trained in procedures relating to confidentiality such that no identifying information will be released without the participant's consent.

Subject records will be imaged into an electronic format or stored in a locked cabinet inaccessible to staff other than the PI and the Program Director, in a locked room; records will include the subject number but no other identifying information.

The databases are maintained within the medical center firewall. Data that are obtained and/or stored with OSU will be maintained in confidence according to the rules and regulations of the Arthur G. James Cancer Hospital and the Richard J. Solove Research Institute, The Ohio State University, and HIPAA requirements.

Data will continue to be securely stored until all analyses and manuscripts have been completed. The IRB at OSU will be notified of any intent to destroy data.

Information obtained during this study will not be placed in the participant's medical record.

Laboratory Data. Electronic and paper laboratory data will be stored and safeguarded as outlined above. Laboratory personnel performing the assays will be blinded to the status of the sample.

Information Warehouse (IW). The IW is a comprehensive informatics platform supporting basic, clinical, and translational research. Date from the IW is verified against standard codes and values to check for its validity. The IW adheres to OSUMC data security policies which safeguard patient data against outside intrusions. While protected by the enterprise wide security measures, the IW data is further secured by the implementation of its own security procedures. Users of the IW are assigned distinct roles that allow them to access the portion of the data that they have permission to. This includes permissions to de-identified area only to ensure HIPAA compliance. Data obtained from the IW will be maintained as described above.

Social Media. Data collected from the landing page associated with the social media recruitment approach will be encrypted and stored in a PHI Tier 4 approved database within the OSUCCC Marketing consumer website, which is maintained by OSU IT and behind the OSUMC firewall.

vi. Will data be sent outside of OSU

Coded or de-identified data may be provided to researchers or laboratories involved in this study outside of OSU via data sharing agreements. Laboratory or other study data will not be included in the subject's medical record.

vii. Quality Control Procedures

Study documents. To maintain control over the quality of staff activities and data capture, the Program Director will periodically conduct observational visits and review all subject forms. The Program Director will debrief the staff and discuss solutions to any challenges or quality control issues that arise. If issues continue to arise, more frequent observations will be conducted. As needed throughout the study, the Program Director will conduct conference calls or in-person visits to provide additional guidance or instruction to field staff.

Throughout the recruitment period, staff will collect and keep close inventory of consent forms and payment receipts, reviewing the same to verify that they are completed accurately and completely. If necessary, incomplete forms will be sent back to staff to remedy. Physical procedures and specimen collection performed by study staff will be observed by the Program Director periodically. Bronchoscopies and phlebotomy procedures performed by trained staff (e.g., Drs. Tsai along with nurses) will continue to receive the typical oversight provided in their general role (i.e., OSUMC).

e. Specimens

i. Specimens to be collected

Lavage fluid and brushing samples via bronchoscopy will be collected. Urine, blood, nasal brushing, nasal lavages and saliva will also be collected.

ii. How the specimens will be collected or accessed

The bronchoscopy procedure and specimen collections are described above. Specimens will be collected by trained staff and medical personnel participating before and during the bronchoscopy.

At the time of collection, each specimen is labeled with a unique barcode label. The label will contain a specimen identification number that will easily identify each specimen type and be electronically linked to the subject study ID. The specimen specific barcode that is printed on the labels will also be found on the specimen collection form. In order to maintain confidentiality, no other identifiable information will be placed on the samples. The labels, suitable for low-temperature freezing, will be placed on the collection tubes/containers and on any freezer cryovials. To minimize the chance of mislabeling a specimen, the identification numbers will be preprinted on the labels and placed on the specimen collection forms. Data for tracking specimens (e.g., date collected, subject study number, specimen identification number, date received, condition of specimen, laboratory sent to for analysis, and date sent to laboratory) will be kept in a secure centralized database.

Only authorized study personnel will have access to the biospecimens. Specimens will be coded and subject identifiers will not be available to laboratory staff.

iii. Specimen collection timeline

Specimens will be collected at baseline bronchoscopy (day1) and follow-up bronchoscopy (~day 71).

iv. How the specimens will be stored and protected

Biospecimens will be processed and aliquoted by the Shields' laboratory staff. Specimens will be stored until needed for analysis in -80° freezers in the Shields' freezers located at 2001 Polaris Parkway, Columbus, OH. A biorepository (separate protocol) will be created for future studies. The Shields' laboratory freezers are currently maintained by the OSU Biorepository and Biospecimen Resource. Samples are stored in locked freezers, which are only accessible to authorized study personnel and facility management. The temperature of the freezers will also be continuously monitored by an electronic monitoring system and routinely monitored by the biorepository personnel to ensure the integrity of the samples. A specimen collection form will accompany the specimen throughout the processing activities; times/dates processed will be documented on the form. The freezer facility is managed consistent with the National Cancer Institute Biorepository Guidelines.

Specimen tracking data (e.g., date collected, subject study number, specimen number, date received, condition of specimen, location in the freezers, laboratory sent to for analysis, and date sent to laboratory) will be kept in a secure centralized database. The password protected database will be maintained on a secure computer that is only accessible to authorized study staff. The computer will be stored in a locked room. Any paper copies of reports or copies of paper-generated lab results will be coded and stored in a locked file cabinet or imaged to an electronic format and stored behind a firewall on a backed-up, password-protected server. Only approved study personnel will have access to this information.

v. Who will have access to the specimens

Only approved study personnel (e.g., interviewers, laboratory personnel, and biorepository personnel) will have access to specimens.

vi. Will specimens be sent outside OSU for analysis

Specimens may be shared for analysis with researchers or laboratories involved in this study outside of OSU.

f. Human Subjects Information

i. Potential Risks

Participation in this study is voluntary. While completing the survey at baseline and follow-up, there is the minimal risk of mental discomfort. Participants will be instructed that they can refuse to answer a particular question if they feel uncomfortable or end the questionnaire altogether.

As COVID-19 or other viral illnesses persist, study participation may increase a subject's risk to increased viral exposure. According to the CDC, signs and symptoms associated with COVID-19 are: fever or chills; cough; shortness of breath or difficulty breathing; fatigue; muscle or body aches; headache; new loss of taste or smell; sore throat; congestion or runny nose; nausea or vomiting; and diarrhea. Severe conditions that may occur if infected with COVID-19 include: trouble breathing; persistent pain or pressure in the chest; new confusion; inability to wake or stay awake; and bluish lips or face.

There are some known risks associated with bronchoscopy, which is a routine test performed on patients with a variety of lung diseases and other co-morbidities. Major risks are extremely rare [62], and the procedure is frequently performed on critically ill patients or those with significant lung disease. Risks include minor bleeding in the airway, rare collapse of a part of the lung requiring a chest tube (no biopsies will be taken), temporary lowering of blood pressure, temporary alteration of heart rhythm, and temporary narrowing of airways due to airway muscle spasm, airway injury, dental/vocal cord injury, fever, infection, pneumonia, blood clots, and death (rare in persons without reactive airway disease). Minor risks also include the discomfort associated with the passage of the bronchoscope. Any discomfort is minimized by local anesthetics given before and during the procedure. Other side effects can include allergic reactions to drugs, a cough, sore throat, or mild hoarseness for several hours thereafter, although these routinely resolve by the next day. However, occasional patients have been reported to have temporary fever following bronchoscopy. Transient lowering of blood oxygen levels may occur, and extra oxygen may be given as determined by the physician. There are no known long-term hazards of washing a small area of the lung with sterile salt water, and there is no known danger of altering the lung's normal functions. As a precautionary measure the volunteer's heart rhythm and rate, blood pressure, and oxygen level will be monitored during the procedure.

Under OSU-2015C0088, "Effects of Electronic Cigarette Use on the Human Lung" (PIs: Shields and Song), we have conducted over 100 bronchoscopies. One subject had the procedure aborted prior to the insertion of the bronchoscopy due to an incidental arrhythmia noted on heart monitoring before the procedure. One procedure was halted due to patient discomfort. There have not been any significant adverse reactions.

PFT, exhaled CO and FeNO measurements are not invasive procedures, and they are safe for most people. Risks include dizziness, shortness of breath, or coughing. There are no known risks associated with providing a urine sample. Risks for the nasal brushing and lavages are minor and may include discomfort associated with rubbing the brush against the inside of subject's naris, coughing or gagging, local burning feeling with eye tearing, and minimal local bleeding. These symptoms are temporary and should diminish within a few minutes. Bleeding is rare considering the bronchoscope is larger in diameter than the nasal brush. A small needle will be placed in an arm vein for administering medication during the procedure, if the subject consents to this. There are minor risks associated with receiving an IV during the bronchoscopy or blood draw right before the bronchoscopy procedure. These may include transient pain upon insertion of the needle. A small bruise might develop at the site of needle insertion. A transient lightheadedness may develop and on rare occasions and fainting may occur. The volunteer must have a friend accompany them home and they must not drive, as they may feel slightly dizzy from the anesthetics or sedatives (i.e., Versed).

Use of e-cigs poses minimal risks that are generally short-lived such as throat irritation, dry cough, mouth irritation, nausea, headache, and dizziness, vertigo or light-headedness. Sore throat, dry mouth, shortness of breath and mouth ulcers have also occurred, but they are much less common. Patients will be informed to stop using their e-cig and to contact study staff if they experience pallor (pale appearance), cold sweat, nausea, salivation, vomiting, abdominal pain, diarrhea, dizziness, disturbed hearing or vision, tremor, mental confusion, or weakness, as these may be symptoms of a nicotine overdose. If they are unable to contact us, then they will be advised to stop using the e-cig until their contact. For severe symptoms, they will be advised to seek prompt medical attention. Cartridges for the e-cig are sealed; however, if cartridges were to leak, nicotine-containing liquid could pose a risk for poisoning, especially to children and pets. If stored improperly, overheating, fire, and/or explosion of the device may occur, leading to burns and possibly death. Risks related to long-term use of e-cigs are unknown.

As of 10/28/19, the Centers for Disease Control and Prevention (CDC), the Food and Drug Administration (FDA), and several states and federal health departments are investigating a multi-state outbreak of severe lung disease associated with using e-cigarette/vaping products, mostly in THC users. The CDC and FDA are investigating what, if any, are the commonality of the illnesses, and what e-cig device designs or e-liquid contaminants may be causative. As of 12/20/2019, the FDA and CDC https://www.cdc.gov/media/releases/2019/p1220-cases-EVALI.html recommend that people should not:

- Use e-cigarette, or vaping, products that contain THC.
- Buy any type of e-cigarette, or vaping, products, particularly those containing THC from informal sources like friends, family, or in-person or online dealers.
- Modify or add any substances to e-cigarette, or vaping, products that are not intended by the manufacturer, including products purchased through retail establishments.

The implication is that these new reports represent some recent change in devices or how they are used, and that existing commercial products are not associated with these serious and rare respiratory effects.

Risks for phlebotomy are considered minimal and limited to bruising and minor bleeding. Some subjects may experience vaso-vagal type symptoms.

Risk associated to varenicline include: Nausea, abnormal (e.g., vivid, unusual, or strange) dreams, constipation, flatulence, and vomiting are the most common adverse reaction. Dr. Wewers or other MD associated with the study will review the reported nausea symptoms and provide additional instructions as needed (e.g., dose reduction). Other side effects reported after using varenicline include: (1) neuropsychiatric such as changes in mood, psychosis, hallucinations, paranoia, delusions, homicidal ideation, aggression, hostility, agitation, anxiety, and panic, as well as suicidal ideation, suicide attempt, and completed suicide; (2) seizures; (3) interaction with alcohol; (4) cardiovascular events; (5) somnambulism (sleepwalking); (6) angioedema and hypersensitivity reactions; (7) serious skin reactions; and (8) accidental injury.

Subjects will be instructed to discontinue varenicline, contact study staff and immediately seek medical care if they experience any of these symptoms. Dr. Wewers or other MD associated with the study will review the reported symptoms and provide additional instructions (e.g., dose reductions) as needed.

NRT products are safer than cigarettes. The negative health effects of cigarettes are proven. Of the 4,000 chemicals found in tobacco smoke, over 60 are known to cause cancer. By using NRT to quit smoking subjects can reduce exposure to many chemicals found in tobacco smoke. Risks related to the use of NRT, which is an over-the-counter sold medication, are minor. It should be noted that the dosage delivered from NRT or combination NRT is generally less than levels delivered from smoking.

NRT package insert information on side effects:

Lozenges - The side effects that most commonly occur are hiccups, mouth or throat irritation, heartburn, or other stomach problems such as nausea. The patient will be informed to stop using the product and contact study personnel if they experience mouth problems, persistent indigestion or severe sore throat, irregular heartbeat or palpitations, nausea, vomiting, dizziness, diarrhea, weakness, or rapid heartbeat.

Gum - The side effects that most commonly occur are hiccups, mouth or throat irritation, heartburn, or other stomach problems such as nausea. The patient will be informed to stop using the product and contact study personnel if they experience mouth, teeth or jaw problems, irregular heartbeat or palpitations, nausea, vomiting, dizziness, diarrhea, weakness, rapid heartbeat, or symptoms of an allergic reaction (such as difficulty breathing or rash).

Patches - The side effects that most commonly occur are headache, nausea, upset stomach, and dizziness. The patient will be informed to stop using the product and contact study personnel if they experience irregular heartbeat or palpitations, nausea, vomiting, dizziness, weakness, rapid heartbeat, skin redness caused by the patch that does not go away after four days, or if your skin swells, or you get a rash. If the patient experiences vivid dreams or other sleep disturbances, the patient may remove the patch at bedtime and apply a new one in the morning.

ii. Protections Against Risk

As COVID-19 or other viral illnesses persist, study procedures have been updated to adhere to institutional standards and best practices. Given the situation continues to evolve, study procedures may also adapt to address subject and personnel safety as well as study and institutional resources. Staff will implement precautions to mitigate viral exposure. Such precautions may include limiting physical interactions, when possible (e.g., remote/telephone visits or physical in-person distance), assessing signs and symptoms of viral illness (e.g., temperature and exposure assessments), and providing appropriate personal protective equipment (PPE) to subjects and staff (e.g., masks, gloves, googles, face shields, etc.). Participants may be asked to undergo COVID-19 testing prior to the bronchoscopy procedures. Bronchoscopy procedures as well as the PFT and measurement of exhaled CO and FeNO may be conducted in a negative pressure bronchoscopy suite. If warranted, the study may be temporarily suspended if there is significant community spread and resources (e.g., bronchoscopy staff or space) are limited. Specimens will be collected by trained staff using Universal Precautions for Prevention of Transmitting Bloodborne Pathogens.

The main risks associated with the study are related to the bronchoscopy procedure. While risks are possible, bronchoscopy is a routine procedure that is performed on a variety of subjects, including those with significant medical problems. The bronchoscopy and brushing procedure will be performed by trained personnel. Monitoring of heart activity (EKG, electrocardiogram), pulse oximetry, and breathing is performed continuously throughout the procedure by nursing staff and other health professionals. In the case of an emergency, pulmonary and/or critical care board-certified physicians trained in advanced care for critically ill patients are immediately available and can perform life-saving care if needed, as would be typical for patients in the hospital. Subjects are provided with post-bronchoscopy written instructions that also include phone numbers to call with issues after bronchoscopy, such as respiratory complaints.

Staff will do their best to ensure privacy.

For individuals recruited by subjects, the RDS approach is structured to protect potential subjects' privacy because individuals are provided a referral code or coupon. The seed/respondent can choose to follow-up with the study or not and there is no expectation of the individual beyond meeting the basic criteria (current smoker, age 21 or older, willing to be screened). In other words, the seed is not required to enroll in the study, which minimizes potential coercion. Furthermore, staff will not confirm the identity or status of individuals who may have been provided RDS codes/coupons. Referring individuals to the study is optional. Subjects' participation in the study will not be affected if they do not invite others or if those invited do not meet the criteria outlined above.

Information about the project is distributed through passive and active routes. Passive recruitment similarly respects potential subjects' privacy - individuals who see information about the study (e.g., flyers, ads) can choose to follow-up with the study or not. For active recruitment (e.g., staff approaching potential subjects in public spaces), we train staff to give information (i.e., approved recruitment materials) to individuals at a given location without targeting by race, age, or other characteristics. Individuals who are interested can ask follow-up questions if they desire. If any individual requires information in a more private environment, staff look for a quiet, private location locally if possible, or schedule a time to speak at more length in a way that protects privacy.

Throughout the study, including at consent, if a subject is accompanied by a guest, he or she will kindly be told that to protect the subject's privacy, he or she will need to step out of the room. If the subject voluntarily states they would like the guest to remain, the research staff will document the occurrence in the progress notes. Visits will occur in private rooms. If study tasks need to be completed in common areas, staff will ensure the area and interactions are as private as possible.

Study subjects' confidentiality will be maintained at all times. The subject's name will not appear on any specimens and the subject's participation will remain confidential. Subjects will be assigned unique study identification number (study ID). All study data will be identified by study ID only. The use of a computerized tracking system will enable authorized study personnel to link participants and specimen numbers. Any electronic information (e.g., questionnaire data, laboratory data, tracking systems, etc.) will only be accessible to authorized study personnel who have the necessary password(s). All computer systems will be password-protected against intrusion; all network-based inter-site communications of confidential information will be encrypted. Access to computer-stored information will require simultaneous knowledge of the data format, computer language, file name and passwords.

Safety measures will be implemented to minimize the risk of breaching confidentiality. The autonomy of participants will be protected by informing all participants of the purpose of the study, and allowing them to opt out of participation at any time without repercussion. Only authorized study personnel will have access to data identifying individuals. Data from questionnaires, physiologic measurements, and the various laboratory and data analyses will be stored in password secured databases (under control of the data manager). Data provided to statistician for analysis purposes will not include identifiers. Access to information linking identifiers with the unique IDs will be restricted to the study manager and to others on a very strict need to know basis.

Any paper documents with identifying information (e.g., informed consent forms) will be kept in a locked cabinet inaccessible to staff other than authorized personnel or imaged to an electronic format and stored behind a firewall on a backed-up, password-protected server.

If participants are asked questions that they that they do not wish to answer for any reason, they will be told that they do not have to respond to that question. Every effort will be made to ensure that the safety and confidentiality of each participant is maintained and that they do not incur any undue risk. Participants have the right to withdraw from the research study at any time they so desire.

The principal investigator and the research team will not tolerate any employee coercing an individual into joining a research study. Appropriate corrective action will be taken against any member of the research team who uses coercion or undue influence in order to recruit participants into a research project.

Only study investigators and staff who have completed their institutional or the NIH human subjects training will be able to work on this project. Documentation of completion will be maintained on file at OSU.

Extensive efforts will be made to protect personal information to the extent allowed by law; however, absolute confidentiality cannot be guaranteed.

Providing e-cigs to subjects may increase lung injury including pulmonary toxicity. If subjects continue to use vaping device/e-cigarette products, they will be informed of the potential health risks. They will also be instructed

to only use those products provided by the study, and to avoid modifying or adding any substances including THC. Subjects will be instructed to promptly seek medical attention and contact study staff if they experience unexpected symptoms (e.g., cough, shortness of breath, chest pain, nausea, vomiting, diarrhea, abdominal pain, fatigue, fever, weight loss) or have any health concerns. Subjects can also call their local poison control center at 1-800-222-1222. We will be asking subjects about their health at every study visit and ask them to call us if they experience any of the health issues described.

Providing e-cigs to subjects may entice them to use e-cigs after the trial. The importance of smoking cessation will be reinforced. Subjects are informed about the dangers of smoking at entry and exit to the study, and they are entering the study with the knowledge that we do not understand the long-term dangers of e-cigs. Nonetheless, we will contact subjects three months after the trial to determine if they are using any type of tobacco product, including e-cigs, and again reinforce complete cessation. All subjects will be encouraged to contact the QUITLINE.

While varenicline is an FDA-approved smoking cessation medication that is well-studied and the side effects well described, subjects with adverse signs or symptoms suspected to be related to Chantix (varenicline) will be evaluated by one of the physicians involved in this study. Participants will be instructed to reduce the amount of alcohol they consume until they know whether varenicline affects them. Participants will be instructed to notify study staff of new or worsening CV symptoms and to seek immediate medical attention if they experience signs and symptoms of myocardial infarction (MI) or stroke. Participants will be instructed to discontinue varenicline and contact study staff immediately at first appearance of skin rash with mucosal lesions. Participants will be instructed to use caution driving or operating machinery until they know how varenicline may affect them.

While NRT is an FDA-approved smoking cessation aid that is well-studied and the side effects well described, subjects with adverse signs or symptoms suspected to be related to NRT use will be evaluated by one of the physicians involved in this study (e.g., Shields, Tsai) and dose adjustment, reduction or discontinuation will be recommended as appropriate. Patches are available in 21mg, 14mg, and 7mg. Gum and lozenges are available in 4mg and 2mg.

Research results will not be provided to participants.

Data Monitoring for Participant Safety: See attached DSMP.

iii. Benefits

The subject will receive no benefit other than financial compensation for participating in this study. In general, this study will provide a benefit to future e-cig users as the findings will be reported to the FDA and inform policy, both for reducing toxicity (if any) and to identify harmful effects for consideration as a potential cessation device. The risk for the subject is no greater than a patient of bronchoscopy in a clinical setting. The OSU Wexner Medical Center's standards of care for this procedure are followed. Research results will not be returned to the subject.

iv. Reporting of adverse events

See attached DSMP.

v. Premature Removal of Participant

See attached DSMP.

vi. Study Termination Procedures

The Ohio State University has the right to suspend or permanently close the research study at any time.

vii. Completion of study

A participant is considered to have completed the research study when he or she completes all data collection and specimen donation, and their follow-up bronchoscopy. They will be called at three months after the second bronchoscopy procedure to assess their recent tobacco use history and health status.

g. Statistical Methods

i. Data Analysis Plan

The analytical plan does not change due to the increase in intervention period from 4 to 8 weeks.

Descriptive statistics and clustering (e.g., principal components analysis [PCA]), will be performed for all biomarker data measured at the different visits. Baseline data (1st bronchoscopy) will then be compared between the four groups (3 conditions and control) using a one-way analysis of variance (ANOVA). The non-parametric Kruskal-Wallis test (continuous or ordinal variables) or the chi-square test (categorical variables) will be applied when the normality assumption of the data is not met. We will compare controls to complete substitution with the SREC (with and without nicotine) or NRT conditions. Generalized linear models (GLM) will be employed with measurement (cell count, gene expression, etc.) as the dependent variable, a covariable for baseline measure, and a main effect of arm. Other relevant variables that are associated with the biomarker results, e.g., baseline smoking, FTND, or gender will also be included in our models as they are potential confounders. We also will assess batch effects and include batch as a co-variable in the model (all samples will be analyzed at the same time, except for cell counts). We plan to use baseline molecular measurement as a covariable rather than modeling the change between baseline and follow-up because the former is more powerful [300]. Given the large dynamic range of the cell count and the 'omics data, we will initially consider a normal distribution. If the data are determined to deviate from normality, we will log-transform the data or assume a non-normal distribution of residuals (such as Poisson) for the GLM. Urine PG will be measured at the 2nd bronchoscopy visit and compared to baseline to verify e-cig use. As an exploratory analysis, urinary PG will be used as the exposure predictor variable assessing the biomarker results as dependent variables. Urine anatabine, urine anabasine and nicotelline will be used to confirm complete substitution or a predictor variable for smoking reduction. The cotinine level will be used as a surrogate for smoking reduction.

The 'omics' (miRNA, mRNA, proteomics and metabolomics) analysis and visualization of the data will be performed in the R statistical language. Data will be log2-transformed and normalized using either quantile normalization for gene expression (miRNA and mRNA) or TIC/MSTUS normalization for metabolomics [301]. Unsupervised clustering analysis, including PCA and hierarchical clustering will be performed to visualize natural clusters in the dataset and evaluate data quality. Multiple testing corrections using the Benjamini and Hochberg False Discovery Rate (FDR) will be performed [302]. Significantly altered genes and metabolites will be defined as those with FDR corrected p-value<0.05 and fold changes >2 or >1.5, respectively. Identified genes or metabolites will be used to identify relevant networks and pathways using Ingenuity Pathways Analysis. For gene expression, we will further study associations between miRNA-mRNA pairs based on publically available target prediction databases such as TargetScanHuman, miRDB, miRWalk2.0 and others [303-308]. Global integrative analysis, through correlations, will be performed using Partek software.

Understanding the epigenomic/genomic/proteomic/environment context and regulation of metabolic phenotypes will expand our knowledge of the effects of e-cig on lung biology, and could contribute to finding successful interventions, including accurate predictions of e-cig related adverse effects. Typical omics integration methods include logistic regression or correlations among omics measurements (when measured in the same samples), intersection of gene/protein/metabolite lists (when measured in different samples), or further downstream integration by assessing alterations in pathways [309-311]. Because our omics measurements will be made in the same sample, we will be able to directly assess relationships between our molecular data through regression or correlation analyses. Of note, typical regression/correlation approaches are not appropriate for low sample numbers, and they do not readily adjust for differences in sample size between phenotype groups or for potential confounders. Another approach, which we propose here, is to directly test whether associations between miRNA or gene or cytokine and metabolite levels are specific to a group assignment. To test this, we will apply a novel linear model approach recently developed by Dr. Mathe for these types of analysis: m = g + t + g:t, where m are metabolite abundances, g are miRNA or gene or cytokine levels, t is treatment group (e.g. SREC vs. continued smoking), and g:t is the interaction between miRNA or gene or cytokine level and treatment group. A statistically significant g:t interaction p-value would indicate that gene (or miRNA or cytokine):metabolite relationships are present in one treatment group and not the other. Pathway enrichment analysis of associated miRNA, genes,

cytokines, and metabolites (resulting from the linear models) will be performed in Ingenuity Pathway Analysis to pinpoint altered pathways and better understand how the epigenomic/genomic/proteomic/environment affects metabolic phenotypes associated with e-cig usage. Furthermore, miRNA, genes, cytokines, and metabolites involved in treatment-specific associations will be used to predict treatment group, in an effort to produce an e-cig inflammation signature. Ten-fold cross-validations of various linear-based and non-linear based machine learning methods will be tried (e.g. Random forests, naïve Bayes, Lasso) and the resulting specificity and sensitivity will be assessed to select the best performing model. Of note, the cross-validation will help ensure that the predictive models are not overfit and could be predictive on new validation data. The contribution of input molecular measurements (gene, cytokines, miRNAs, metabolites) to the model will be assessed to define the minimal set of features that yield the optimal cross-validated models.

All statistical analysis and visualization of differential methylation will be performed using Partek Genomics Suite (Partek). Raw intensity data (idat files) will be imported into Partek and normalized using the Subset-quantile Within Array Normalization (SWAN). Any probes with detection P > 0.05 and probes in Y- chromosome to avoid sex-specific methylation bias will be filtered out before further analysis. To identify differentially methylated CpGs, methylation β-values will be converted to M-values by logit-transformation for the statistical modeling purpose. For preliminary identification of patterns in DNA methylation, unsupervised hierarchical clustering among the groups of samples will be performed. The Euclidian distance among the groups of samples will be calculated by the average linkage. In order to assess variance among samples, Principal Component Analysis (PCA) will be done. To characterize the methylation patterns, differentially methylated CpGs will be classified by enhancer, CpG island or its neighbors [2 kb regions upstream and downstream of the CpG islands (shores) or 2 kb regions upstream and downstream of the CpG island shores (shelves)] and functional promoters [within 1500 bp of a transcription start site (TSS) (TSS1500), within 200 bp of a TSS (TSS200), 5' untranslated regions (5'UTR), first exon (1stExon)], and other regions (body, 3'UTR, or intergenic). Separately, we will also analyze for DNA methylation levels of miRNAs promoter regions to investigate if miRNAs methylation appears to be altered between the groups. When we have a full sample, multiple testing corrections will be performed using the Benjamini and Hochberg False Discovery Rate (FDR with significantly differential methylation levels defined at corrected P <0.05. Genes corresponding to differentially methylated CpGs will be used for identification of gene networks and biological pathways using the Ingenuity Pathways Analysis. In addition, we will examine differences in methylation for candidate genes that have been previously shown to be associated with lung cancer and/or with smoking using the same methods. To understand the potential biological contribution of differentially methylated CpGs to coding gene or miRNA expression (from on-going pilot study), we will correlate between the differentially methylated CpGs (M-value) from the EPIC methylation array and matched coding gene or miRNA expression (log2 transformed intensity) from the Affymetrix Human Transcriptome array or Affymetrix GeneChip miRNA array using Spearman correlations. To determine if differences in DNA methylation are associated with cytokines levels (as measured for IL-6, IL- 10, TNF-alpha, and TNF-alpha in BAL in the current on-going study), we will select CpGs corresponding to IL-6, IL- 10, and TNF-alpha from the EPIC methylation array (targeted analysis) and correlate between methylation levels and corresponded cytokines. Correlations controlling for possible confounders (e.g., age) will be explored using multiple linear regression models. We will also explore if the correlation between methylation and cytokine levels may be mediated by gene expression of cytokines using interaction analysis.

Study staff and biostatisticians will monitor the success of RDS and incorporate appropriate analyses to address bias if appropriate.

ii. Sample Size and Power

We will enroll 128 subjects (32 per group). For power calculations, we will use a planned 66% retention rate at 5 weeks, based on the clinical trial discussed above, and knowing that we have a 100% retention for never smokers receiving serial bronchoscopies. Thus, the final sample size will be 21 subjects per group (n=84) For the purposes of sample size considerations, we will consider cell counts based on the current available data in the cross-sectional study comparing smokers to e-cig users, as indicated above. Based on our current data of smokers undergoing bronchoscopy, the mean cell count for the smokers was 2.41E+07 (1.48E+07) and the e-cig users was 4.03E+07 (4.84E+07). In the preliminary cross-sectional study, we see decreases in cell counts of

~2.5 fold with e-cig use. For our power calculations we will assume a CV = 0.26 (from the healthy smokers undergoing bronchoscopy). For power, because of the large number of tests (the total number is currently unknown because of unknown numbers of metabolites and gene expression passing filtering), we will assume a significance threshold that corresponds to one expected false discovery per 10,000 tests (0.0001). Thus, for a final sample size of 21 in the control group and 21 in each use condition, for a two sided analysis, we have 80% power to detect ≥1.5-fold difference in cell counts/cytokine levels between each condition and controls for a two-sample t-test. For gene expression/metabolomics/proteomics data, with the same considerations except a CV=0.5 (approximate from many gene expression data sets with which we have worked), we have 80% power to detect ≥2.1-fold differences.

To assess changes of mtDNA genetic features (mutations by next generation sequencing and copy numbers) in the bronchial and nasal epithelium of randomized smokers, arms will be compared pairwise. To correct for multiple comparisons, we will assume a significance threshold corresponding to one expected false discovery per 100 tests (p=0.01). Biomarkers will be examined for comparisons between pre and post in each visit for bronchial and nasal samples separately. Generalized linear models (Poisson [mutation] and Gaussian [CN]) will be employed with feature as the dependent variable, a covariable for baseline measure, and a main effect of Arm. For CN, with 21 samples per group, considering a significance threshold of 0.01, a two-sided, two-sample t-test achieves 80% power to detect an 1.4 fold difference between arms considering a coefficient of variation of 0.32 [298]. For mutation, Poisson regression achieves 80% power to detect a mutation rate ratio of 2.7 between arms considering a significance threshold of 0.01 and 21 samples per group [299].

To investigate if changes in mtDNA alterations are associated with lung inflammation and changes in other omics features, generalized linear models (Gaussian) will be employed, with follow-up inflammatory markers and gene expression as the dependent variables, covariables for baseline levels and arm, and changes of mtDNA features (ΔmtDNA =Follow – Baseline) as the independent variables. Because of the number of genes that will be tested, for power we will consider a significance threshold corresponding to one expected false discovery per 10,000 tests (p=0.0001). With 84 samples, considering a significance threshold of p=0.0001 and unit standard deviations for dependent variable residuals and ΔmtDNA features, linear regression achieves 80% power to detect a slope of 0.48.

Follow_Expression = Baseline_expression + arm + ΔmtDNA feature + e.

Significant effects of mtDNA feature will indicate that changes gene expression (or inflammatory markers) are associated with changes in mtDNA feature while accounting for between arm effects.

To compare mtDNA alterations of smokers between bronchial and nasal samples, for mutational burden, a generalized linear mixed model (Poission) will be employed with a random effect for subject, and a main effect for location (bronch vs. nasal). Poisson regression achieves at least 80% power to detect a mutation rate ratio of 2.1 between the sample origins considering a significance threshold of 0.01 and 84 paired samples. For mtDNA CN, a generalized linear mixed model (Gaussian) will be employed with a random effect for subject, and a main effect for location (nasal vs. bronch). A two-sided paired t-test achieves 80% power to detect a mean of paired differences of 0.4 standard deviations of the paired differences considering 84 paired samples and a significance threshold of 0.01. Correlations (CN) and Cohen's kappa (mutational burden) will be calculated between the two tissues for each of the assays.

We will also compare complete substitution with the SREC use with continued smoking or NRT use. Generalized linear mixed models will be employed with measurement (mtDNA genetic features) as the dependent variable, a random effect for patient, covariables for baseline measure and sample origin, a main effect of arm, and an interaction effect of origin* arm. This analysis will investigate whether treatment is associated with differences between tissues.

Feature = sample&rand + baseline + origin + arm + arm*origin + e.

The interaction term will be comprised of four groups of 21 for each pair of arms. To conservatively estimate the power to detect this interaction effect, the power will be calculated while adjusting the significance threshold for the number of pairwise tests (n=6; p=0.01/6=0.0017). A two-sided, two-sample t-test achieves 80% power to detect an 1.5 fold difference between any two groups considering a significance threshold of 0.0017, 21 samples per group, and a coefficient of variation of 0.32 [298].

As an exploratory analysis, we will study dose-response associations of cigarette smoking with biomarkers at baseline bronchial and nasal samples separately, generalized linear models (again, Poisson or Gaussian) will be fit with biomarkers as the dependent variable and covariates (e.g., cigarettes per day [cigs/day] and urinary cotinine levels, separately), adjusting for confounders, such as sex and age. THC use will also be examined.

h. Data Safety Monitoring Plan

See attached.

5. References

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