In-vivo Effects of E-cigarette Aerosol on Innate Lung Host Defense

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Complete Title: In-vivo effects of E-cigarette aerosol on innate lung host defense

Drug or Device Name(s): Cinnamaldehyde, a flavor in e-cigarette liquids

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ABBREVIATIONS AND DEFINITIONS OF TERMS

Abbreviation	Definition
AE	Adverse Events
ATS	American Thoracic Society
BP	Blood Pressure
CA	Cinnamaldehyde
CEMALB	Center for Environmental Medicine, Asthma and Lung Biology
Co57	Cobalt 57
CTCAE	Common Terminology Criteria for Adverse Events
EMS	Emergency Medical Services
EENT	Ear, Eyes, Nose, Throat
FVC	Forced Vital Capacity
FEV1	Forced Vital Capacity in one second
HR	Heart Rate
HS	Hypertonic Saline
IS	Induced sputum
KeV	kilo electron volt
LAIV	Live Attenuated Influenza Vaccine
MMAD	Mass Mean Aerodynamic Diameter
MCC	Mucociliary Clearance
mCi	millicurie
NHLBI	National Heart, Lung and Blood Institute
NIH	National Institute of Health
NNAL	(4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol)
NSAIDS	Non-steroidal anti-inflammatory drugs
PE	Physical Examination
PHI	Personal Health Information
ROS/RNS	Reactive Oxygen Species/Reactive Nitrogen Species
RR	Respiratory Rate
RT-PCR	Reverse Transcription – Polymerase Chain Reaction
SABA	Short Acting Bronchodilator Agent
SI	Sputum Induction
SOP	Standard Operating Procedure
SpO2	Peripheral capillary oxygen saturation
Tc99m-SC	Technetium99m Sulfur colloid
uCi	microcurie
UP	Unanticipated Problem
ELF	Epithelial Lining Fluid

PROTOCOL SYNOPSIS

Study Title:	In-vivo effects of E-cigarette aerosol on innate lung host defense
Funder	NIH
Clinical Phase	Pilot
Study Rationale	While e-cigs are commonly represented as safer alternatives to tobacco cigarettes, little is known regarding the health effects of their short- or long-term use. The responses and the e-cig components exerting these effects on the airways are largely unknown. Hence, a critical need currently exists in identifying e-cig components, namely specific flavors, modifying respiratory immune responses.
Study Objective(s)	**Primary** • Determine the effects of CA-containing e-cigarettes on airway epithelial cell ciliary function (i.e. MCC) in vivo **Secondary** • Baseline differences in MCC in non-smokers/non-vapers as compared to e-cigarette users • Differential cell counts in CA-induced changes in IS samples • Regional lung clearance rates via MCC scan.
Test Article(s) *(If Applicable)*	An e-cigarette that contains CA-containing flavors.
Study Design	This will be a blinded, crossover study to assess the effect that vape liquid containing cinammaldhyde (CA) has on the innate mucosal immune system of current users of e-cigarettes. The primary endpoint will be the effect of the CA-containing e-cigarette liquid on airway epithelial cell ciliary function, in vivo. The primary analysis will be achieved via the mucociliary clearance (MCC) scan. Prior to enrollment, subjects will be screened based on their ability to produce sputum with sufficient viable cell counts for analysis as well as their history of radiation exposure. Study visits thereafter will include a baseline visit for both the vape/non-vape cohorts to measure baseline MCC, epithelial lining fluid (ELF), induced sputum (IS), blood values, and urine. The e-cigarette cohort will have a total of seven visits, and the control cohort with 3 visits.
Subject Population	**Inclusion Criteria** **1.** Subjects age 18-40

key criteria for Inclusion and Exclusion:	**2.** Subjects who currently use a vaping device and an equal number of subjects who do not use a vaping device **Exclusion Criteria** **3.** Any pre-existing lung disease (asthma, cystic fibrosis) **4.** Any significant chronic illness, such as, but not limited to, heart disease, uncontrolled hypertension, diabetes, auto-immune disease **5.** Any use of tobacco products (other than e-cig) in the past 3 months, or a greater than 10 pack year history of smoking cigarettes. **6.** Pregnant or nursing women **7.** Subjects with a history of radiation exposure in the past year which exceeds annual safe limits.
Number Of Subjects	**Total Number of Subjects:** 54 total, 22 who currently vape, 32 who do not vape (data from n=12 subjects of this group already exist from a previous study)
Study Duration	Each subject's participation will last up to 3 months. There is a 2-6 week washout each session. The entire study is expected to last up to 3 years.
Study Phases **Screening** **Study Treatment** **Follow-Up**	**(1) Screening:** After informed consent is obtained, a medical history, including medication use and radiation history, will be obtained. Spirometry will be collected, and subjects will undergo sputum induction procedure. Subjects must produce an adequate sputum (based on cell counts and viability) in order to continue in the study. **(2) Intervention:** Subjects who successfully complete all screening procedures will return to the study lab and undergo an ELF collection, blood, urine, and MCC/sputum induction. Subjects will return approximately. 24 hours later for a follow up MCC scan to undergo sputum induction again. At this point, participation ends for all non-vaping subjects. Subjects who vape will continue their visit with a training session with the lab's e-cigarette device, as well as their own. Subjects will return within 2 to 6 weeks and repeat the vaping session, with randomized e-liquid. Subjects will then undergo MCC scan followed by epithelial lining fluid (ELF) collection and induced sputum. Subjects will return the next day for a 24 hour follow up scan, epithelial lining fluid (ELF) collection, blood draw, urine collection, and sputum induction. There will a 2-6 week washout, and the subject will return to repeat the session, vaping the randomized e-liquid.
Efficacy Evaluations	NA
Pharmacokinetic Evaluations	NA
Safety Evaluations	Subject safety is monitored throughout the study with vital signs and spirometry, as well as urine pregnancy testing prior to each MCC scan.

Statistical And Analytic Plan	Refer to section 5.2
DATA AND SAFETY MONITORING PLAN	**Describe who is responsible for data quality management and ongoing assessment of safety: PI, independent medical monitor, internal safety committee, or DSMB.** Case report forms to collect data will be developed by the study team. Identifiable data (name, contact information) is kept on a form separate from the research data, however all forms will have the study number. All paper data will be maintained by the study coordinator or the investigators, and will be kept in locked offices during non-business hours. REDCap is trackable by ONYEN, access to identifiers will only be given to those who need it to do their job. Subjects are monitored in real time during all procedures, and an MD is always available during the inhalations of the e-cig and for the TC99 inhalations. An MD is also immediately available for all sputum induction procedures. The PI will be responsible for the data management, and the PI, along with a study physician, will be responsible for the subject safety assessments.

1 BACKGROUND AND RATIONALE

"Vaping" flavored electronic cigarettes (e-cigs) is gaining popularity in the U.S., particularly among teens and young adults. Availability of different flavorings is often cited as the main reason among youth/adolescents to try e-cigs, yet the recent Surgeon General's report focused on nicotine addiction as the main health concern. There are currently more than 7,700 commercially available flavored e-cig liquids (e-liquids), which contain food-safe flavorings without significant data on potential inhalation toxicity. Common e-liquid flavorings contain aliphatic aldehydes for fruity flavors and α,β-unsaturated aldehydes, such as cinnamaldehyde (CA), for spicy flavors. Spicy or cinnamon-flavored e-liquids are popular and frequently identified as "Best Sellers" by e-liquid retailers.

While e-cigs are commonly represented as safer alternatives to tobacco cigarettes, little is known regarding the health effects of their short- or long-term use. We have previously demonstrated that cigarette smoking significantly affects innate immune function of the respiratory mucosa by modifying mucosal immune cell function, immune gene expression, and mucociliary clearance (MCC), thereby potentially enhancing susceptibility to respiratory infection. We have previously demonstrated that suppression of immune gene expression was significantly greater in the upper airways/nasal mucosa of e-cig users as compared to non-smokers and cigarette smokers, but the mechanisms mediating these responses and the e-cig components exerting these effects are largely unknown. Hence, a critical need currently exists in identifying e-cig components, namely specific flavors, modifying respiratory immune responses.

We will use innovative translational research models to examine how one leading popular flavoring component, CA, modifies respiratory mucosal immune responses. Yet, how CA could affect respiratory immune responses *in vivo* in humans is unknown, which is the overall objective of this study. The feasibility of our proposed

studies is supported by preliminary data demonstrating that CA 1) inhibits immune cell function in vitro and reduces ciliary beat frequency on airway epithelial cells in vitro. Thus, based on these data we hypothesize that **CA-containing e-liquids suppress innate mucosal immune function in humans in vivo.** We will use integrated translational research models and our unique capability of assessing MCC and induced sputum airway immune cells in humans *in vivo* for this study.

Introduction

1.1 Name and Description of Investigational Product or Intervention

Each vaping session will occur with a vaping device (The Vapor Shark DNA 250), which allows manual control and recording of the vapor settings (voltage, wattage, puff volume, and puff frequency), using the myVapors™ software. Data can be directly imported into a database, thus allowing detailed information of a very controlled vaping session. To reduce variability, subjects will be asked to use the e-cig device (The Vapor Shark DNA 250) and e- liquids provided by us for the indicated vaping sessions. Please note, we are not studying the device, we are simply using it to deliver the e-liquid in a controlled fashion.

Subjects will vape with two different e-liquids (one completely devoid and one containing at least 30mM CA similar to the commercially available "Hot Cinnamon Candies" flavor. These e-liquids will be purchased from a local vaping shop.

Non-Clinical and Clinical Study Findings

-Potential Benefits: There are no direct benefits to the subject. Society benefits by understanding potential adverse health effects induced by exposure to e-cigarette aerosols containing specific flavoring chemicals.
-Risk /Benefit Assessment: The radiation exposure is within safe limits for healthy adults. The effective dose for the three measures of MCC (including Co57 transmission scans) is approximately 133 mRem. This is less than the natural environmental radiation that adults receive every year, which in Chapel Hill is about 300 mRems. The risk from the radiation dose received from this procedure is too small to be detected. Urine pregnancy tests will be done prior to each measurement of MCC as there is no safe risk to a fetus for this investigational study. Women with a positive pregnancy test, or those who do not use a reliable form of birth control will not be included. Spirometry and sputum induction carry a risk for wheezing, which is uncommon in healthy individuals. Only subjects who currently use an e-cigarette will be recruited for the active part of the study, e-cig naïve individuals will not be intentionally exposed. Despite the investigational nature of this study, the risk of the 3 separate vaping sessions are not more than the risk subjects take when they vape outside this study.

1.4 Relevant Literature and Data

Recent studies demonstrated that e-cig vapor exposure decreases antimicrobial and phagocytic function of immune cells resulting in reduced bacterial clearance and increased bacterial burden in the lung. Aldehydic flavoring compounds, such as CA have reported immunomodulatory function in immune cells, such as macrophages (Macs), including anti-inflammatory properties, decreased ROS/RNS formation, and alteration in immune cell surface marker expression. Most of these studies were conducted in non-human cell lines without direct relevance to the lung and did not reveal biochemical mechanisms mediating these effects. In our lab we recently demonstrated that Macs obtained from healthy volunteers via bronchoalveolar lavage (BAL) and exposed to the CA- containing e-liquid "Sini-cide" ex vivo almost completely blocked phagocytosis and the ability to engulf BioParticles® (Clapp et al., 2017). In addition to "Sini-cide", we screened popular e-liquids purchased from a local vape shop and found additional e-liquids containing between 900µM-1M CA ("Kola", "Hot Cinnamon Candies", and "Sini-cide"). Exposure of Macs to other CA-containing e-liquids ("Hot

Cinnamon Candies") also decreased phagocytic function in Macs, a response that could be mimicked by treating cells with CA alone in a dose-dependent manner, indicating that CA alone has direct inhibitory effects on Mac phagocytosis at levels that are not cytotoxic (Clapp et al., 2017). Other components of the upper respiratory tract that are essential innate defense mechanisms include the mucosal barrier and mucociliary clearance (MCC). In MCC, motile cilia beat in a coordinated fashion to move airway mucus toward the larynx. This complex process relies on the structure, number, and coordinated movement of airway cilia. Dysfunction of the coordinated beating of cilia and impaired MCC is associated with significant airway disease. Our preliminary data demonstrate that exposure of differentiated well-ciliated human bronchial or nasal epithelial cell (HBECs or HNECs) to e-liquids containing the flavoring chemical cinnamaldehyde (CA) ("Sini-cide"), or CA alone causes acute, yet reversible, ciliary stasis. Hence, acute exposure of CA-flavored e-cigs impairs ciliary beating, which could translate into significant effects on MCC in humans in vivo.

2 STUDY OBJECTIVE

The overall objective of the study is to determine the effects of CA-flavored e-cigarettes on MCC and airway immune cells in vivo. To achieve this objective, the study will address the following specific aims:

Aim 1. Characterize and compare two cohorts (current non-vaping controls and current e-cig users) in terms of baseline characteristics and measures of MCC and measures of immune cell function.
Aim 2a. Evaluate the effects of CA on ciliary function in terms of measures of MCC in current e-cig users.
Aim 2b. Evaluate the effects of CA on airway immune cells obtained through induced sputum (IS) in current e-cig users.

3 INVESTIGATIONAL PLAN (brief overview)

3.1 Study Design

We will evaluate the acute effect of CA-flavored e-cigs on MCC and IS immune cells in up to 22 healthy, young adults who are current e-cig users with a total of less than 10 pack-years cigarette smoking history. MCC will be measured by gamma scintigraphy at baseline and following controlled vaping of e-liquids with and without cinnamon flavoring. Two different e-liquids (one completely devoid and one containing at least 30mM CA similar to "Hot Cinnamon Candies" which is commercially available) will be used for two separate randomized vaping sessions. The randomization scheme for the two different e-liquids (e-liquids with and without CA) will be generated by using the Web site Randomization.com (http://www.randomization.com), assigned treatment Regimen A and B by an assigned study team member, and provided to the study team. This individual will also be responsible for loading the e-cigarette with the appropriate solution for that session prior to the vaping sessions. n

We will also recruit non-vaping control subjects (n=22), who will only undergo the baseline testing and thus serve as a non-exposed/non-vaping control group. We will aim to recruit similar numbers of males and females in both cohorts. While we cannot guarantee age-matching and sex-matching in these cohorts, based on our previous studies (Martin et al., 2016), we do not expect to find significant age and sex differences in the two cohort. In addition, potential confounders, such as age, sex, and BMI will be included as covariates in our multivariate analysis. Observations obtained from the non-vaping control group will provide necessary information on potential baseline differences in the two cohorts (i.e. current vapers versus non-vaping controls). These data from the non-vaping control group are important to provide a reference for any potential CA-induced changes in the vaping group. Hence, there are two stages of the study:

Stage 1. A cross sectional observational cohort comparison of baseline MCC and IS immune cells in a reference cohort of n=22 non-vaping control subjects and E-cig cohort of n=22 currently vaping subjects (confounding based on other variables such as BMI, sex, age is possible for this stage).

Stage 2. A randomized comparison of changes in MCC and IS immune cells after Regimen A (e-cig us without CA) and Regimen B (e-cig use with CA). The cohort of e-cig users will undergo a randomized 2-treatment, 2-period, 2-sequence crossover study of CA exposure.

For stage 1, baseline measurements of Tc99m-SC clearance will be used to measure each subject's normal baseline MCC and IS immune cell characteristics. For both stages, subjects will be asked to complete a vaping diary to record information on the device and e-liquids (name/vendor/e-liquids/puffs/device settings) used during their normal vaping sessions for the entire duration of the study. In addition, for stage 2, subjects will be asked to maintain their current habits for the duration of the study, not to significantly increase or decrease their vaping patterns, including the nicotine concentrations of their e-liquids

For stage 2, for each e-cig vaping session (Training and MCC Test Days), subjects will be asked to follow a laboratory-based protocol involving 6, 5-minute paced vaping segments (1 puff/minute) over a 1 hour time period, vaping the e-liquid with and without CA provided by us. On each Test Day, subjects will undergo the vaping protocol immediately prior to inhalation of the Tc99m-SC (10 min between end of vaping and inhalation of Tc99m-SC). An initial deposition scan of Tc99m-SC will then be obtained followed by dynamic imaging of the lung with subjects seated in front of the gamma camera to determine potential changes in MCC induced by acute exposure to CA-flavored e- cigarettes. Induced sputum samples will be collected at baseline, and after each MCC scan, and app. 24 hours after completion of the MCC scans.

The two randomized vaping sessions will be separated by 2-6 weeks. While there are no data providing specific information on the duration needed to washout the effects of CA on MCC, previous studies examining changes in MCC following inhalation of other aerosols have shown that this washout period is sufficient to prevent potential carryover between the two treatments (Burbank AJ, 2017).

3.2 Allocation to Treatment Groups and Blinding (if applicable): The e-liquid will be randomized for either CA containing flavoring or non-CA containing fluid. Each vaping subject will crossover at the second session. Subjects and as well as personnel involved in the MCC and other analyses will be blinded to the CA or non-CA.

3.3 Study Duration, Enrollment and Number of Subjects 22 e-cig users, and 22 non-e-cig users will be recruited into the observational phase (stage 1) of the study. Each subject will have a screening visit followed by a baseline visit with an MCC scan. After the baseline/MCC scan visit, the subject will return within approximately 24 hours for a follow up MCC scan and a sputum induction. The non-e-cig users are completed at this point (note: data from 12 previously studies non-e-cig users will be included in the overall data analysis). The subject in the e-cig cohort will return the next day for a follow up scan/sputum induction as well as a training session with our e-cigarette device/lifeshirt. There will be a 2-6 week washout and then the vaping/MCC/SI session. The entire study should be concluded in no more than 3 years.

3.4 Study Population

-Inclusion and Exclusion Criteria

Inclusion:

- Subjects who currently use a vaping device and an equal number of subjects who do not use a vaping device
- Age 18-40
- Subjects must have a FVC and FEV1 of at least 80% of predicted. Subjects who fall out of the normal range will be offered a copy of the test to share with their personal physician.

Exclusion:

- Any pre-existing lung disease (asthma, cystic fibrosis, etc.)
- Any significant chronic illness, such as, but not limited to, heart disease, uncontrolled hypertension, diabetes, auto-immune disease
- Any use of tobacco products (other than e-cig) in the past 3 months, or a greater than 10 pack year history of smoking cigarettes.
- Pregnant or nursing women
- Subjects with a history of radiation exposure in the past year which exceeds annual safe limits.

STUDY PROCEDURES (what will be done)

Screening/Baseline Visit procedures:

Subjects will arrive for the screening and baseline visit. After informed consent is obtained, a medical history, including medication use and radiation history, will be obtained. Urine pregnancy tests will be obtained on all women, and must be negative. Urine will be collected for measures of cotinine and NNAL (both markers of nicotine). Spirometry will be collected, and subjects will undergo SI procedure. Subjects must produce an adequate sputum (based on cell counts and viability) in order to continue in the study. Subjects who vape will be given a daily diary to record information on the device and e-liquids (name/vendor/e-liquids/puffs/device settings) used during their normal vaping sessions for the entire duration of the study.

Subjects who successfully complete all screening procedures will return to the study lab and undergo a baseline MCC, 40cc of blood collection, epithelial fluid lining collection (ELF), urine collection, and an induced sputum (IS) procedure. Subjects will be asked to refrain from vaping for 12 hours prior to the MCC study. Subjects will return 24 hours later for a 30-minute follow up gamma camera scan and sputum induction Vital signs will be collected prior to and immediately after sputum induction. At this point, participation ends for all non-vaping subjects.

For those who vape, at this point we will introduce the device and instruct the subject in the proper technique. Before the training session begins the subject will be fitted with a Lifeshirt vest (Vivometrics, Inc.) that will allow continuous measurements of breathing pattern (tidal volume, respiratory rate, flow, etc.) by respiratory inductance plethysmography. Measurements will be verified with the TSI Series 4000 mass flowmeter; this is a passive measurement that occur concurrently with the subject's inhalation. Pulmonary mechanics will be measured while the subject vapes with our device as well as their own e-cigarette device

The Vapor Shark DNA 250™ and e-liquids are provided by us for the indicated vaping sessions. The subjects will be asked to follow a laboratory-based protocol involving 6, 5-minute paced vaping segments (1 puff/minute). This device is used allows manual control and recording of the vapor settings (voltage, wattage, puff volume, and puff frequency), using the myVapors™ software. Data can be directly imported into a database, thus allowing detailed information of a very controlled vaping session.

3.5

Intervention/Treatment procedures (by visits):

For stage 2, subjects who are continuing in the study will return to the lab 2-6 weeks later for the MCC/vape session. Subjects will be assigned a treatment regimen based on the randomization scheme produced by the Study Coordinator. Subjects will be asked to refrain from vaping for 12 hours before this session. After vital signs, baseline spirometry, urine, and ELF collection, subjects will vape with the randomized e-liquid, either with or without CA. Ten minutes after completion of the vaping session, subjects will undergo MCC scan for 120 minutes followed by immediately by vital sign collection, ELF collection, spirometry, and IS. Vitals signs will be collected again prior to discharge. Subjects will return the next day for a 24-hour follow up scan followed by vital signs collection. Another IS, ELF, Urine, and blood collection will be conducted in the post-MCC/vape session. Vital signs collection will be repeated prior to discharge. There will a 2-6 week washout, and the subject will return to repeat the session, vaping the crossover e-liquid.

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(2) <u>Intervention</u>: study intervention/experimental treatment.

Cinnamaldehyde (CA)-containing e-cigarettes versus e-cigarettes with other flavors, such as tobacco.

3.4 Follow- up procedures (by visits) Each subject will be seen 24 hours after the vaping session for a 30 minute retention scan, and a SI. There is no other planned follow up.

3.5 Unscheduled visits: Any subject who has an AE or UP related to the study will be asked to return to the research lab for evaluation. This would include vital signs collection, spirometry, and exam by a study physician. Any AE that is not related to the study must be resolved such that it will not interfere with the data collection, or impact the subject's safety (such as an upper respiratory infection).

3.6 Concomitant Medication documentation Concomitant medication use will be collected at the screening visit and updated at each visit. There are no medications given in this study.

3.7 Rescue medication administration (if applicable) Albuterol is always available for rescue in subjects who undergo pulmonary research studies and procedures. Only subjects who currently vape will be recruited into the active part of the study, and known lung disease is an exclusion from participation.

3.8 Subject Completion/ Withdrawal procedures: There are no planned completion /withdrawal procedures. Subjects who withdraw will be replaced.

3.9 Screen failure procedures All procedures will stop once it is determined that a subject has screen failed. Subjects will be notified of any abnormal clinical finding (increased blood pressure, abnormal spirometry, for example).

4 STUDY EVALUATIONS AND MEASUREMENTS (how measurements will be made)

- **List variables that will be abstracted from medical charts**: No information will be abstracted from the subjects' medical record. Subjects will be interviewed to collect health history.
- **Describe baseline evaluation**: Vital signs, including heart rate, respiratory rate, blood pressure and oxygen saturation, as well as lung auscultation, will be collected as described in the sections above. Temperature will be measured only at the beginning of each study day. Spirometry is collected based on ATS recommendations and standards.

Describe how measurements will be taken.

Spirometry:

This test measures the volume of air that can be exhaled and the rate of airflow during exhalation after a maximal inhalation. Subjects will inhale as deeply as possible, then exhale as rapidly and completely as possible into the spirometer. Measurements obtained from each maneuver include the forced vital capacity (FVC), the forced expiratory volume in the first second (FEV1), the maximal mid-expiratory flow rate (FEF 25-75%) and the peak flow (PF). The largest FVC and FEV1, from at least 3 acceptable trials, are selected for analysis; the flow rates are selected from the trial with the largest sum of FVC. Spirometry will be performed following ATS recommendations.

Hypertonic Saline Induced Sputum procedure:

FEV_1 and forced vital capacity (FVC) are measured to determine the post bronchodilator baseline FEV, and FVC values. The FEV_1 values that match a 10% and 20% fall from post bronchodilator baseline are determined. An ultrasonic nebulizer is filled with 20ml of 3% hypertonic saline (inhalation grade for respiratory use only, 3% NaCl) to begin the test. The nebulizer is set to the maximum output setting and turned on. The subject is instructed to seal his/her lips around the nebulizer mouthpiece and breath normally (i.e., tidal breaths) for 7 minutes. The saline is nebulized through the mouthpiece in a jet stream and inhaled. The nose is not occluded for this procedure. The subject is encouraged to come off the mouthpiece at any time to cough if a sputum sample from the lower airways (i.e. not from the back of the throat) is ready for expectoration. Prior to expectoration, subjects are asked to blow their nose, rinse their mouth with water, and clear their throat to avoid the inclusion of non-airway fluid samples.

The sample is expectorated into a sterile specimen jar and capped. Following the measurement of FEV_1 after the first 7-minute inhalation period, the concentration of saline is increased from 3% to 4%, provided the FEV_1 falls by < 10% from the post-bronchodilator value. A volume of 20ml fills the nebulizer well on each occasion. If the FEV_1 falls by between 10-20% of the post bronchodilator value, the test proceeds but the concentration of saline remains the same If the FEV_1 falls by > 20% or if troublesome symptoms occur, the nebulization is discontinued and albuterol is available if necessary to relieve symptoms. Troublesome symptoms included cough, chest tightness, and general discomfort. The same procedure is followed for the final 7 minute inhalation period using 5% hypertonic saline provided the FEV_1 safety parameters described above have been

met. The nebulization is stopped after 21 minutes, or earlier if a sputum sample of good quality is obtained (i.e. visible sputum plugs).

Sputum plugs will be processed as described by us before to obtain airway cells and cell-free sputum supernatants (Lay et al. 2011). IS cells will be analyzed for surface markers and immune cell function (i.e. phagocytosis and respiratory burst) using flow cytometry, which will be expressed as mean fluorescence intensity (MFI) as described by us before (Lay et al., 2011). Immune mediator levels will be assessed in the cell-free sputum supernatants using commercially available assay kits and expressed as pg/ml cell-free sputum supernatant (Lay et al., 2011).

Vital signs are collected before and after sputum induction procedures.

MCC procedures:

Prior to the each MCC study a transmission Co57 scan will be performed to define the lung boundaries, to assign regions of interest, and to normalize these regions for lung volume differences. A rectangular phantom containing the radioisotope Co57 (< 25 mCi) will be placed in front (5cm) of the subject sitting with his/her back to the gamma camera for 30 seconds. The transmission scan has been used by us (e.g. 05-2358, 08-0795, 06-1016, 13-1605, 15-938) and others to provide a delineation of lung boundaries for assessing regional deposition /clearance of the inhaled radioaerosol. Prior to the transmission scan on each study day we will place 2 spot markers of Americium241 (0.9 microcurie (uCi) each, gamma 66 KeV) on the upper and lower back of each subject during scanning (both Tc99m-SC deposition/retention and Co57 transmission). With dual isotope imaging, these spot markers will allow alignment of images for more accurate determination of regional deposition/retention. These very low radiation sources have been obtained from commercially available home smoke alarms. The placement of these markers will be determined to be outside the lung field during the transmission scan. Their location will be marked in semi-permanent ink for later placement during Tc99m deposition/retention scans. The shielded side of this source will be placed/taped onto the subject's skin.

Radiolabeled Tc99m-sulfur colloid will be delivered using a modified Pari-LL nebulizer (MMAD 9.5 um). This is a closed delivery system that produces 80 ml/sec air flow, and therefore limits the inspiratory flow rate to this value. While seated in front of a gamma camera subjects will perform single inhalations lasting ~10 seconds each from the delivery system, and will exhale at 500 ml/sec (using feedback from a flow meter in the breathing circuit). Approximately 5 of these inhalation maneuvers will be required to deposit an adequate isotope dose to the lung. Subjects will be allowed to breathe normally (off the nebulizer) in between each inspiratory maneuver. Each volunteer will practice these maneuvers prior to the actual radioaerosol inhalation to guarantee his/her proficiency. The activity of Tc99m-SC loaded in the nebulizer will be adjusted to provide an estimated 40 uCi deposited in the lung for each MCC scan. A single crystal detector will be placed at the subject's back during inhalation to monitor dose to the lung. Total inhalation time should be less than 5 minutes in all cases.

Immediately following isotope inhalation, the subject will gargle and drink water to clear activity that deposited in the mouth into the stomach. The subject will then (within a minute of final inhalation maneuver) be seated in front of a large-field-of-view gamma camera to begin acquiring particle retention images. Serial, 2-minute gamma images will be captured continuously for the first 34 minutes following isotope delivery. Thereafter, 2 consecutive 2-minute images will be obtained at the start of every 10-minute period until 2 hours post isotope inhalation. The subject will also return the following day to obtain a scan of 24-hour lung activity.

Pulmonary Mechanics:

We are interested in recording breathing patterns associated with subject vaping on our standard e-cigarette device. These data may be useful for estimating the dose of inhaled e-cigarette particles and vapor for a given individual. Before the training session begins the subject will be fitted with a Lifeshirt vest (Vivometrics, Inc.) that will allow continuous measurements of breathing pattern (tidal volume, respiratory rate, minute

ventilation, flow, etc/) by respiratory inductance plethysmography. The LifeShirt is a lightweight (8 oz.), machine washable shirt with embedded sensors. Respiratory function sensors are woven into the shirt around the patient's chest and abdomen. The LifeShirt Recorder™ is a mobile device that continuously records and stores the subject's physiologic data on a compact flash memory card. The data can be imported into the VivoSense software for analysis. There is no risk associated with this non-invasive measure and has been successfully used in previously IRB approved studies (10-0556 and 05-1644).

Airflow data for each puff taken during the vape session will be collected in real-time using a TSI Series 4000 mass flow meter and LabVIEW software. During the inhalation of each puff, inspired air will be directed through the mass flow meter and a 0.2um filter in line with but prior to the e-cigarette device. Airflow data will be recorded at 0.01 second intervals for the duration of a user-defined puff to determine the exposure dose of each inhaled puff. This method provides 1) an accurate means for recording puff topography and exposure dose, and 2) validation for puff data collected with the LifeShirt. This measurement is passive/non-invasive and does not contribute to any form of risk in this investigation.

Nasal epithelial lining fluid (ELF) collection procedure:

ELF is obtained by spraying the nostril with 0.9% sterile, normal saline irrigation solution (about 100 µl per nostril) one time per nostril. One 10x55mm strip of filter paper, cut from Leukosorb paper (Pall Scientific, Port Washington, NY) on a laser cutter, is inserted into the anterior part of the inferior nasal turbinate of each nostril. The nostrils are clamped shut using a padded nose clip for two minutes. Strips are then removed from the nostril and collected in 1.5ml tubes. They are then stored in strips in a -20°C freezer until elution.

Describe rating scales, tests, psychological tools, laboratory evaluations, etc. NA

4.1 Efficacy Evaluation (if applicable)

Not applicable

4.2 Pharmacokinetic Evaluation (if applicable) NA

4.3 Safety Evaluations Spirometry is monitored throughout the sputum induction procedure as the most common complication is decreased FEV1. The subjects FEV1 must return to at least 95% of baseline prior to discharge. Vital signs are measured before and after each sputum induction. Urine pregnancy tests are done prior to each MCC session.

5 STATISTICAL CONSIDERATION

Provide sufficient detail to permit assurance that the sample size is justified and the statistical methods are sufficient and appropriate for the research question(s).

Primary Endpoint

The primary endpoint will be CA-induced changes in MCC as compared to the baseline measurement.

The right lung will be used to analyze both regional deposition and MCC to avoid interference from stomach activity when imaging the left lung. Central (C) vs. peripheral (P) deposition will be determined by regions of interest (ROI) analysis and normalized to C/P from a Cobalt57 transmission scan. The whole lung ROI bordering the right lung will be used to calculate whole lung retention (Rt) (corrected for decay and background) as a fraction of the initial counts in the right lung, over the 120 minute clearance period at 10 minute intervals (two-2 minute images summed for each 10 minute time point, e.g. images 1 and 2 for initial time 0 and images 6 and 7 for time 10 minute). For each Rt vs. time data set, the average MCC (or 100* (1- Rt) over the 120 minute period of observation) will be computed (i.e. average of the 10 minute clearance values from 10 to 120

minutes). This calculated MCC represents the average clearance, as a percentage, at the midpoint of the 10-120 minute retention vs. time observation.

5.1 Secondary Endpoint

Secondary endpoints include:

- Baseline differences in MCC in non-smokers/non-vapers as compared to e-cigarette users
- Differential cell counts in CA-induced changes in IS samples.
- Regional lung clearance rates via MCC scan.

5.2 Exploratory Endpoints

Exploratory Endpoints include:

- Inflammatory Cell Expression in Epithelial Lining Fluid.
- CA-induced changes in immune cell function
- Peripheral Blood Mononuclear Cell Analysis
- Expression of inflammatory mediators in Blood Serum
- Pulmonary Mechanics with subjects own e-cigarette device
- Pulmonary Mechanics with investigator e-cigarette device
- CA-Induced changes in Epithelial Lining Fluid
- Peripheral blood mononuclear cell analysis

5.3 Statistical Methods

Repeat measures of MCC in healthy nonsmokers have provided paired standard deviation data that establish confidence we can detect a significant change in MCC associated with the e-cig challenge. Similar to our previous studies (Bennett et al., 2014), repeat measures analysis will be used to assess differences in MCC between the three study days to determine CA-induced changes within each study subject. For the stage 1 studies, comparisons by ANOVA will also be done for baseline MCC values obtained in non-e-cig users each study day, to detect potential overall differences in MCC between e-cig users and non-users (both smokers and non-smokers). To control for confounding variables, such as age, BMI, and sex, we will include these variables as covariates in our multivariate analysis, similar to our previous study (Martin et al., 2016). Statistical analyses will be conducted in close collaboration with Dr. Haibo Zhou, Professor in Biostatistics, who leads the Biostatistic Core for the Center for Environmental Medicine Asthma, and Lung Biology (CEMALB), where this study will be conducted.

All statistical estimates (e.g. treatment effects, means, medians, mean differences, etc.) will be tabulated along with corresponding confidence intervals. Dr. Zhou, the leader of the biostatistics core for the CEMALB will lead the statistical team overseeing data analysis and guide the statistical analysis for this study. For all analyses described above a p-value of ≤ 0.05 is considered to be statistically significant. For data not statistically significant based on these criteria, they will be reported as “inconclusive”.

For the Stage 2 studies, linear model methods for repeated measures will be used to evaluate the effect of CA-containing e-liquids on MCC and IS endpoints (e.g., immune cell function and inflammatory mediators).

For each outcome variable (e.g., total MCC) the dependent variable will be the post-vaping change from the baseline value that was obtained during the screening visit.

Models for MCC measures. For total MCC scores in Stage 2, $\Delta Y_{ij}(t)$ is the change-from-baseline score in the k-th period (k = 1, 2) from the i-th participant in the j-th treatment sequence (j = A:B, B:A). The primary analyses will focus a linear mixed-effects model for $\Delta Y_{ij}(t)$ conditional on the following explanatory variables: assigned treatment regimen, and period. For MCC scores, the model is

$$[\, \Delta Y_{ij}(t) \,|\, \underline{X} \,] = \mu_A X_1 + \mu_B X_2 + \pi X_3 + \lambda S X_4 + \lambda_A X_5 + \beta \{Y_{ij}(t_0) - Y(t_0)\} + \varepsilon_i + \varepsilon_{ij}(t)$$

in which carryover effects λ_A and λ_B are assumed to be zero in the target population. The expected values are shown in the table below.

Entries are expected value — Treatment Sequence

	A:B	B:A
Period 1	μ_A	μ_B
Period 2	$\mu_B + \pi$	$\mu_A + \pi$

This linear mixed-effect model will be fitted separately for each of the outcome variables of interest. For each of these the fitted model will be used to tabulate statistical estimates (all with 95% confidence intervals) of the population parameters of interest which include total variance (σ^2), intra-class correlation (ρ), the direct effect of treatment ($\mu_A - \mu_B$), and the period effect π. The interpretive analyses of the results will focus on the point estimates and confidence interval estimates of the population parameters of interest: μ_A , μ_B, $(\mu_A - \mu_B)$, π, σ^2, ρ.

Sensitivity analyses. To guide our level of trust in these main results, sensitivity analyses will then be performed to evaluate the robustness of the results to reasonable perturbations of the methods and assumptions used. These sensitivity analyses will include use of the post-vaping MCC score as the dependent variable (instead of using change from baseline.) The sensitivity analyses will also include use of a linear mixed-effects models which do not account for period effects as well as versions of the model that account for the baseline response as a covariate:

$$[\, \Delta Y_{ij}(t) \,|\, \underline{X} \,] = \mu_A X_1 + \mu_B X_2 + \pi X_3 + \beta \{Y_{ij}(t_0) - Y(t_0)\} + \varepsilon_i + \varepsilon_{ij}(t)$$

No carryover effects. It is important to note that the 2-treatment, 2-period, 2-sequence crossover design, CO (2,2,2), is incapable of coping with differential carryover effects and is being used in this study only because we are absolutely confident in our assumption that the carryover effects (λ_A and λ_B) are zero in the target population. If this assumption is incorrect, then the results of the study will be biased. The CO (2,2,2) design provides very poor precision for estimation of differential carryover effect ($\lambda_A - \lambda_B$). Any test of the null hypothesis, "$(\lambda_A - \lambda_B) = 0$", will have very little power in this study.

No tests for carryover effects. A two-stage testing procedure will not be used. (e.g., a test for differential carryover is performed first, and if the test is not statistically significant then the data from both periods are used; else, estimates are obtained excluding period-2 data.) That strategy performs poorly in terms of bias, precision and power, and may yield misleading results. If it is plausible that differential carryover effects may exist in the target population, then the best approach is to employ a sufficiently longer washout interval, or use a study design that can account for carryover effects.

Models for immune cell measures. In Stage 2, for immune cell measures obtained via sputum induction (IS) immediately after and 48hrs after each vaping session, the dependent variable in the linear mixed-effects model will be the post-vaping change-from-baseline value. The model is:

$$E[\ \Delta Y_{ij}(t) \mid \underline{X}\ ,\ Y_{ij}(t_0)\ ] = \mu_A X_1 + \mu_B X_2 + \delta_A X_3 + \delta_B X_4 + \pi X_5 + \varepsilon_i + \varepsilon_{ij}(t)$$

Entries are expected value — Treatment Sequence

		A:B	B:A
Period 1		μ_A	μ_B
Period 2	at 0 hrs	$\mu_B + \pi$	$\mu_A + \pi$
Period 2	at 48 hrs	$\mu_B + \delta_B + \pi$	$\mu_A + \delta_A + \pi$

Transformations. Based on prior publications and previous experience with of the outcome measures of interest, some of the measures are known to follow distributions that are skewed or otherwise not well approximated by Gaussian distributions. Appropriate transformations of those outcome measures will be identified and applied prior to analysis of the data.

Missing Values. To address missing baseline covariate data for the Stage 1 analyses, we may employ multiple imputation methods. For Stage 2, the proposed linear model methods are designed to cope with missing data.

As an alternative analysis approach, we will also employ regression modeling techniques. For this study, we will use a linear mixed model approach that considers the above individual tests in a global, unified way where all data are used at the same time. This method should be more powerful than the two sample t-tests, especially when there is a significant carryover effect. This approach will also allow us to account for the influence of sex, race, and BMI. SAS PROC MIXED will be used to fit the linear mixed model. To address potential missing data, we will employ multiple imputation methods to address this issue when appropriate. All statistical estimates (e.g. treatment effects, means, medians, mean differences, etc.) will be tabulated along with corresponding confidence intervals. Dr. Zhou, the leader of the biostatistics core for the CEMALB will lead the statistical team overseeing data analysis and guide the statistical analysis for this study.

5.4 Sample Size and Power Sample size for MCC: In a study of healthy subjects (n=12), using the same methods described here, we found a standard deviation of the delta between 2 repeat MCC measures of 7 % (SD) in average clearance through 90 minutes (Ave90Clr) (i.e. the average of %clearance ((1-retention) X100) over all 10 min data points from 10-90 min) (Bennett et al 2015). In the same study these healthy subjects were challenged with 7% hypertonic saline in a repeated, crossover design, and we observed a decrease from 16% to 12% Ave90Clr 4 hours after HS treatment compared to baseline (no HS). In another recent study, again using the same MCC methods described here, we found that endotoxin (LPS) challenge in mild asthmatics (n=15) resulted in a slowing of MCC from 19% to 14% Ave90Clr 4 hours post LPS challenge compared to baseline. The primary endpoint/comparison for the current study will be CA e-liquid induced changes in MCC as compared to the baseline MCC measurement (paired comparison). Secondarily the vehicle itself (PG/VG mix without CA, or nicotine contained in the e-liquids) may also modify MCC without CA (also a simple paired comparison). In other words, the e-cigarette without CA may be an active vehicle, not an inactive placebo. If e-liquids with or without CA decrease MCC similarly to either 4 hour post HS or LPS, using an estimated SD of 7.0 % for repeat measures, a decrement of 4.5 % in Ave90Clr (or a 30 % decrement in MCC) may be seen with **n=20** volunteers (β= 0.80 and α=0.05). Based on our in-vitro findings where cilia

were completely immobilized in normal human airway cells for 60 minutes following CA administration, we think the 4.5% reduction in Ave90Clr is likely an underestimate of the anticipated CA-liquid effect. We anticipate a 10% incompletion/drop out rate, which will be addressed by aiming to enroll **n=22** subjects into the study.

The calculated power for the MCC comparison between vapers (e-liquid users) and non-vapers (healthy adult nonsmokers) will be based on the baseline MCC in n=20 for the former and n=32 for the latter (12 subjects studied recently in Bennett et al 2015 and an additional 20 non-vaping subjects here). The proposed difference in MCC (Ave90Clr) between the two groups is 4.5%, a chronic effect similar to the acute hypothesis above and ½ of the relative difference seen between healthy adults and patients with chronic bronchitis with FEV1<40%pred (Anderson et al). From Bennett et al (2015) the mean baseline for health nonsmoking adults was 16% (+/-6). To observe the 4.5 % absolute difference between the two groups we will have a power of 0.75 with a type I error of 0.05. It is unlikely that we will observe the 4.5% change both acutely (discussed above) and chronically, i.e. chronic effects of CA in vapers may mask the effect of a single dose of CA or the baseline MCC may be normal in vapers but slow with each acute dose of CA e-liquids. Studying both the single dose effect within the vapers (repeat comparisons) and the baseline differences between vapers and non-vapers (group comparison) will allow for a more complete picture of e-cigarette effects on MCC. These are the first studies to assess the effect of e-cigarettes on MCC in healthy adult subjects.

To assure that we would have sufficient power to determine statistically significant differences in IS cells in the proposed studies, we used preliminary data obtained from SI immune cells from non-vaping controls and e-cigarette users. In this study, the mean percentage of macrophages with high inflammatory markers in non-smokers was 20.8 with a standard deviation of 24.66. Assuming a difference between baseline and treatment group to be a 2-fold increase in percent macrophages with high inflammatory markers, which we saw in samples obtained from e-cigarette users, we will have a power of 0.965 with a type I error of 0.05 using the sample size of n=20 determined for MCC above.

5.5 Interim Analysis

There is no interim analysis planned.

6 STUDY INTERVENTION (drug, device or other intervention details)

- **Description** The vape device e-liquids will be purchased by the research team, and maintained in a locked file cabinet in the CEMALB. The device will be loaded by study personnel, and the actual vaping session (as previously described) will occur in a supervised area of the CEMALB.
- **Receipt/Storage:** The device and the e-liquids are commercially available and will be purchased by study staff. These items will be stored in a secure area of the CEMALB.
- **Packaging/Labeling:** There will be no labeling attached at study visits. Study staff will log the e-liquid and device use.
- **Dosing** E-liquid with and without CA; tailored to the subjects current nicotine use.
- **Treatment compliance and Adherence**: Subjects will be monitored in real time as the use the vaping devices.
- **Drug Return/Destruction:** NA
- **Drug Accountability:** NA.
- **Radioisotope (Tc99m-SC):** Radiolabeled (Tc99m) sulfur colloid (SC) is prepared using TechneScan SC Kits (Mallinckrodt Medical) with binding efficiencies of greater than 98%. The solution of Tc99m-SC will be

obtained from the radiopharmacy in the Nuclear Medicine Section of the Department of Radiology or from the local Cardinal Health pharmacy.

7 STUDY INTERVENTION ADMINISTRATION(if applicable)

Randomization procedures: Randomization scheme will be generated by using the Web site Randomization.com (http://www.randomization.com) and provided to the study team. An individual not actively involved in the study will maintain the randomization scheme to assure blinding all personnel involved in the study. This individual will assign treatment Regimen A and B and will also be responsible for loading the e-cig with the appropriate solution for that session.

Blinding procedures: The study device will be loaded based on the randomization scheme by CEMALB personnel who are not directly involved in the study procedures or sample analyses to maintain blinding. Logs will be kept to document the order of the e-liquid sessions to assure adherence to the randomization scheme.

Unblinding procedures: Logs will be maintained with e-liquid dispensation, in a secure area. Unblinding will occur prior to data analysis only if there is a medical need.

8 SAFETY MANAGEMENT

-Adverse Event/Serious Adverse Event monitoring procedures Adverse and unplanned events will be assessed at each visits. AE's will be graded per the CTCAE.
- Adverse Event/Serious Adverse Event reporting procedures AE's and UP's will be reported to the IRB using the SOP provided by the IRB. The funding agency will be notified of any SAEs.
-Medical Emergency procedures? The most likely medical emergency would be bronchospasm associated with sputum induction. Albuterol is readily available for rescue treatment if needed, and epinephrine available. There is a code cart in the CEMALB. If more treatment is required, local EMS will be called for transport to UNC Hospitals ED.
-Data Safety Monitoring Plan? Safety is monitored in real time during the study sessions. The PI and a study physician will be notified of any subject safety concerns.

9 DATA COLLECTION AND MANAGMENT

-Monitoring Plan? Data will be entered into REDCap, which is designed for PI investigator-initiated studies such as this one. The PI, Dr. Ilona Jaspers, will be responsible for data management.
-Case report forms? Case report forms will be developed by the study team, using templates from previous studies.
-How will confidentiality be maintained? All paper documents will be maintained by the study staff, and kept in a secure location when not in use.

10 RECRUITMENT STRATEGY: Subjects will be recruited from the existing database at the CEMALB (IRB #98-0799) as well as by mass emails, public advertisements, and the Join the Conquest Website.

11 CONSENT PROCESS

Describe the procedure that will be used to obtain informed consent/HIPAA authorization and assent (if applicable). The study will be described in detail to the subject, including why the study is being conducted, the medication being studies, the risks and benefits, and what is expected of the subject. The subject will be

given adequate time to read the consent, and consent will be obtained prior to any study procedures. We will not seek HIPAA authorization.

-Who will obtain consent/assent? Consent will be obtained by a study coordinator or a study investigator.

-Where will consent /assent process take place? The consent will take place at the UNC CEMALB, located in the Human Studies Facility of the US EPA on Mason Farm Road in Chapel Hill.

-How will investigator assure that subjects comprehend the nature of the study, procedures, the risks and benefits? The subject will be encouraged to ask question regarding the study and procedures. Open ended questions will be asked of the subject to solicit correct responses, to help ensure that the subject understands the study commitment, procedures and risks and benefits.

12 PLANS FOR PUBLICATION

Data collected in the proposed studies will be summarized for peer-reviewed publication. Databases generated as a result of the gene expression analysis will be made publicly available after publication.

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