Human biological responses to low level ozone

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STATEMENT OF COMPLIANCE

The study will be conducted in accordance with the International Conference on Harmonisation guidelines for Good Clinical Practice (ICH E6), the Code of Federal Regulations on the Protection of Human Subjects (45 CFR Part 46), and the NIDCR Clinical Terms of Award. All personnel involved in the conduct of this study have completed human subjects protection training.

SIGNATURE PAGE

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable US federal regulations and ICH guidelines.

Principal Investigator or Clinical Site Investigator:

Signed: _____ Date: _____

Name: David B. Peden, MD

Title: Professor of Medicine

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LIST OF ABBREVIATIONS

AE	Adverse Event/Adverse Experience
ATS/ERS	American Thoracic Society/European Respiratory Society
BAU	Branchial Artery Ultrasound
BP	Blood Pressure
CEMALB	Center for Environmental Medicine, Asthma and Lung Biology
CFR	Code of Federal Regulations
CRF	Case Report Form
ECG	Electrocardiogram
ELF	Epithelial Lining Fluid Collection
EPA	Environmental Protection Agency
FEV1	Forced Vital Capacity in 1 second
FA	Filtered Air
FMD	Flow Mediated Dilation
FWA	Federalwide Assurance
GCP	Good Clinical Practice
HRV	Heart Rate Variability
HIPAA	Health Insurance Portability and Accountability Act
IB	Investigator's Brochure
ICF	Informed Consent Form
ICH	International Conference on Harmonisation
ICMJE	International Committee of Medical Journal Editors
IRB	Institutional Review Board
LV	Left Ventricular
MedDRA®	Medical Dictionary for Regulatory Activities
N	Number (typically refers to subjects)
NL	Nasal Lavage
NEC	Nasal Epithelial Cell Biopsy
NSAID	Non-Steroidal Anti-inflammatory Medications
O3	Ozone
OHRP	Office for Human Research Protections
PHI	Protected Health Information
PI	Principal Investigator
QA	Quality Assurance

QC	Quality Control
SAE	Serious Adverse Event/Serious Adverse Experience
SOP	Standard Operating Procedure
UP	Unanticipated Problem

PROTOCOL SUMMARY

Title:	Human biological responses to low level ozone
Précis:	Subjects will be exposed to low levels of ozone approximating a typical, metropolitan, summer day, ranging from 0.06ppm to 0.08ppm. Subjects will be sedentary, to approximate a typical day. Nasal, pulmonary and cardiovascular endpoints will be measured.
Objectives:	Primary: To explore whether low level ozone exposure, reflective of a typical metropolitan summer day, will cause measurable inflammation in nasal cells or affect cardiovascular endpoints in a sedentary human.
Population:	15 healthy control subjects
Description of Intervention:	Subjects will be exposed to low level ozone, ranging from 0.06ppm to 0.08ppm during the course of the exposure session. Subjects will be sedentary. Nasal, pulmonary and cardiovascular endpoints will be measured.
Study Duration:	60 months.
Subject Participation Duration:	No more than 7 weeks.
Estimated Time to Complete Enrollment:	24 months.

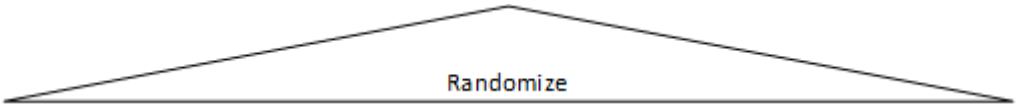
Schematic of Study Design

Prior to Enrollment

Total N 15: All subjects will have completed either CEMALB 98-0799 or EPA 95-0518 prior to invitation into this study. Obtain informed consent. Screen potential subjects by inclusion and exclusion criteria; obtain history, document.

Visit 1

Screening Visit:
Update medical history including medications, collect vital signs including height and weight, urine pregnancy test if indicated, 12 lead ECG, spirometry, venipuncture, physical exam, induced sputum, NEC biopsy. Introduce the subject to the chamber area. Discharge the subject with instructions for the psychological surveys, and with contact information.



Arm 1
Filtered air

Arm 2
O3

Visit 2
Within 6 weeks of visit 1; >3 days after visit 1

Baseline Visit:
Update medical history and medication use, urine pregnancy test if indicated, vital signs. The following samples will be collected / tests performed: ELF collection, NL, NEC biopsy, spirometry, BAU, FMD, LV ultrasound.

Visit 3
Within 2 weeks of visit 2

Exposure visit:
Vital signs will be collected, and subject will be queried for AEs. HRV will be obtained, spirometry performed, O3 monitor placed, telemetry placed, and symptom questionnaire obtained. A 6.5 hour exposure at O3 levels varying from 0.06 to 0.08 with the subject resting or with mild treadmill walking will take place. At the end of exposure the following samples will be collected: Symptom questionnaire, HRV, FMD, LV ultrasound, ELF and NL, spirometry.

Visit 4
24 hours later

Follow up visit:
Subjects will return 24 hours later. Vital signs and AE's will be collected. Then symptom questionnaire, HRV, venipuncture, ELF, NL, Spirometry and sputum induction will be collected.

Visit 5, 6 and 7
6 weeks later
Repeat intervals

Crossover:
Repeat visit 2 as visit 5, visit 3 as visit 6 and visit 4 as visit 7

1 INTRODUCTION: BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE

1.1 Background Information

Air pollutants including ozone have been implicated in affecting health outcomes. In particular, high level ozone exposure has been shown to affect pulmonary function and cause pulmonary inflammation (Silverman, 1976; Hazucha 1987; Devlin, 1991). Troubling community-based work has implicated high ozone levels as being correlated with increased pediatric asthma ED visits (Strickland, 2010). Because of adverse health effects, EPA standards for safe ozone levels have been set, currently at 0.07 ppm (2008). Still, it is estimated that 100 million Americans live in areas where ozone levels periodically remain above the EPA standard. And while this EPA standard had been set based on available data, it remained unclear at the time whether low-level ozone exposure, such as 0.06 ppm, might affect health as well.

Our group previously examined lung function and inflammatory response in adults exposed to low-level ozone, 0.06 ppm exposure for 6.6 hours, while undergoing intermittent moderate exercise (Kim, 2011). We found that in response to low-level ozone exposure (0.06 ppm) with exercise, lung function declines and neutrophilic airway inflammation is observed. What remains unclear, is whether low-level ozone alone – without exercise – will cause similar health effects. If low-level ozone exposure causes measurable decrements in lung function in patients at rest, in a typical, sedentary day, this would be important for human health.

1.2 Rationale

The purpose of this study is to assess the response in healthy adults to low-level ozone exposure (0.06-0.08 ppm) during a 6.6 hour sedentary day. Specifically, we will assess effect of low-level ozone exposure on the following outcomes: changes in profile of nasal inflammatory cells, lower respiratory function, and systemic cardiovascular measures such as heart rate variability, blood pressure, fibrinogen, CRP, and lipid profiles.

1.3 Potential Risks and Benefits

1.3.1 Potential Risks

Ozone exposure:
Exposure to ozone has been studied at our institution and by other investigators and has shown minor effects in lung function in some individuals. Subjects may experience some degree of airway irritation or cough with exposure. These symptoms typically disappear 2 to 4 hours after exposure, but may last longer for particularly sensitive people. In the unlikely event that a subject develops medically significant symptoms

during the exposure period, the exposure will be terminated and the appropriate medical intervention will be provided by the physician on site. Exposure to ozone below 0.08 ppm tends not to cause noticeable effects in most people, and 0.12 ppm is a level many people in the United States may be frequently exposed to, especially in the summer months. Our subjects' maximum exposure will be 0.08 ppm.

Spirometry:
Lung function testing may cause lightheadedness with repeated efforts, but this is unusual. Subjects will be seated in a non-rolling chair, and will be instructed to let us know if they feel at all lightheaded during the breathing test. We would then wait longer between each test in order to prevent lightheadedness

Sputum collection (induction):
Collection of sputum may cause wheezing in some individuals, and coughing repeatedly may make a subject's chest hurt. A physician is immediately available and rescue bronchodilator albuterol is on hand. Subjects will be carefully monitored with pulmonary function testing to ensure a return to baseline before leaving the research lab. Most people can do this test without any problem.

Brachial artery ultrasound:
There are no significant risks associated with ultrasound imaging of the brachial artery, or with the 5 minutes of reduced blood flow that is part of the test. Cutting off blood flow to the arm may result in mild discomfort or temporary sensations of tingling or numbness in the hand until the blood pressure cuff is released. About 1 in 200 patients develop a painless rash on the arm where the blood pressure cuff is placed; this disappears over several days.

Ultrasound of the heart: There is no risk to this procedure.

Blood sampling from vein:
Risks with having blood drawn include mild discomfort or bruising at the site. A small risk of infection also exists. Blood will be drawn by well-trained staff.

Ambulatory ECG monitoring (Holter):
Subjects may have minor skin irritation in the area where the electrodes are attached to the skin. Prior to electrode application, the skin will be treated with alcohol and a cleansing solution. If male, the skin will be shaved in the area where the electrodes are attached. Subjects should not participate if skin is highly sensitive to electrode adhesive or gel. Subjects will not be able to do activities which would result in the monitor getting wet, such as showering or swimming while the monitor is on.

Nasal lavage and ELF procedure: There are no risks associated with the nasal lavage. The ELF can sometime make individuals sneeze.

Nasal epithelial cell collection: This procedure can cause mild temporary bleeding from the nose. It will be treated if it occurs by squeezing the tip of the nose for 5-10 minutes. Nasal biopsy also is transiently painful.

Genetic sampling:
There is risk of discrimination based on results. A Federal law, the Genetic Information Nondiscrimination Act (GINA), generally makes it illegal for health insurance companies, group health plans, and most employers to discriminate against subjects based on genetic information. GINA does not protect subjects against genetic discrimination by companies that sell life insurance, disability insurance, or long-term care insurance. GINA does not protect subjects against discrimination based on an already-diagnosed genetic condition or disease.

Confidentiality: There is risk of breach of confidentiality of medical information and/or data. All individuals who have been granted access to the data to perform their research-related duties will be bound by an agreement of confidentiality. Blood and sputum samples will be stored until all measurements are made for this study. Any samples that are left over will continue to be stored. We will give subjects a consent form which discusses sample storage, titled “Center for Environmental Medicine, Asthma, and Lung Biology Repository for storage of coded samples” (05-2528). Subjects are not required to provide permission for us to store these samples after this study is completed in order to participate in this study.

Psychological questionnaires: It is possible that subjects might find the questions asked in the surveys to be upsetting. These questions are not meant to be intentionally upsetting, but personal questions have a small potential to cause psychological stress. Subjects are not required to answer any questions with which make you uncomfortable.

There may be uncommon or previously unknown risks. You should report any problems to the researcher.

1.3.2 Potential Benefits

There is no direct benefit to the subject. Results from this study may help to determine the effects of ozone on the human airways and cardiovascular system.

2 OBJECTIVES

2.1 Study Outcome Measures

2.1.1 Primary

The primary endpoint is the change in nasal PMN counts, measured in nasal lavage fluid, between FA and O3.

2.1.2 Secondary

Secondary endpoints to be measured include NEC and ELF for inflammatory endpoints, spirometry for change from baseline, change in cardiovascular endpoints FMD, HRV and LV ultrasound in FA vs O3.

Exploratory endpoints correlation of ambient ozone level using a novel, wearable device.

3 STUDY DESIGN

This is a double blind, randomized, crossover study of healthy individuals, who are exposed to relevant levels of ozone vs filtered air, for 6.5 hours and while at rest. This is intended to mimic exposure to ozone levels of typical summer day, on a typical sedentary day.

Multiple samples will be collected, for both nasal and cardiovascular endpoints. Additional information will be collected in for phenotype comparisons.

3.1 Subject Inclusion Criteria

In order to be eligible to participate in this study, an individual must meet all of the following criteria:

- Provide signed and dated informed consent form.
- Willing to comply with all study procedures and be available for the duration of the study.
- Aged 18 to 50.
- In good general health as evidenced by medical history with Vital signs within normal limits on admission to the study: SpO2 $\geq$ 94%, systolic blood pressure between 150-90 mm Hg, diastolic blood pressure between 100-60 mm Hg, afebrile.
- FEV1 of at least 80% of predicted.

3.2 Subject Exclusion Criteria

An individual who meets any of the following criteria will be excluded from participation in this study:

- Medical condition, laboratory finding, or physical exam finding including any chronic medical condition considered by the PI as a contraindication to the exposure study, including significant cardiovascular disease, diabetes requiring medication, chronic renal disease, chronic thyroid disease, or kidney disease, which precludes participation.
- Use of disallowed concomitant medications including systemic or inhaled steroids, NSAIDS or aspirin within 72 hours of dosing, and inability to withhold these medications prior to each session of the study.
- Pregnancy or nursing.

- History of current tobacco use, or more than a lifetime 5 pack year history of tobacco use.

3.3 Strategies for Recruitment and Retention

Subjects will complete either UNC CEMALB screening and database protocol #98-0799 or the Recruitment and Screening of Potential Participants for EPA studies #95-0518 prior to enrollment into this study.

3.4 Treatment Assignment Procedures

A randomization table will be provided by Haibo Zhou, Ph.D, who provides statistical assistance to the CEMALB. TRC maintains the blinding list, and will release it to the study team at the end of the study, or earlier if an interim analysis is done.

3.5 Subject Withdrawal

Subjects may withdraw voluntarily from the study at any time. The investigator may terminate a subject's participation if there is any concern for the health or well-being of the subject, or if the subject fails to follow the study requirements.

3.6 Study Product Description

Ozone is generated by TRC, a contractor for the US EPA. They have procedures in place both for generating and monitoring the O3 concentrations, and they will provide a separate protocol operations plan for this study.

3.7 Accountability Procedures for the Study Product

At the completion of the study TRC will provide a written report for all chamber exposures. The study may be unblinded at the request of the PI or other study medical doctor in the event a subject has an unexpected event and unblinding is necessary for subject treatment or for study continuation.

3.8 Assessment of Subject Compliance with Study Product Administration

Subjects will be queried regarding adherence to medication restrictions.

3.9 Concomitant Medications/Treatments

Subjects will be queried regarding concomitant medication use at each visit. Any new medications will be documented and the PI or covering medical doctor will be notified, and that individual will decide if the subject may proceed with the study.

4 STUDY SCHEDULE

Subjects will arrive for a consent visit and screening for the study. After informed consent is obtained, we will:

1. Collect/update medical history, including any medications
2. Collect vital signs, including pulse, RR, BP, temp, SpO2 and breath sounds.
3. Measure height and weight.
4. Pregnancy test will be done for all women
5. Obtain standard 12-lead ECG for baseline measurement.
6. Perform spirometry according to ATS/ERS recommendations.
7. Perform venipuncture for 50ml of blood for clinical labs, including CBC Diff, chemistries as well as baseline research labs.
8. Perform physical examination (by a study doctor) to evaluate the subject for eligibility.
9. Collect sputum for baseline measurements. Induced sputum is a method for obtaining lower airway secretions. Subjects will be asked not to eat for 2 hours prior to this test as food residue in the mouth may contaminate the samples. Any subject who comes to this study with a diagnosis of mild asthma will be pre-treated with 2-4 puffs of albuterol prior to sputum induction. Subjects will breathe saline from an ultrasonic nebulizer through a mouthpiece for 7 minutes of each saline concentration (3%, 4%, and 5%) while seated. After the first inhalation period subjects will be asked to rinse their mouths out with water, spit into the sink, and then gargle. Then they will clear the throat, and blow their nose. Subjects are then encouraged to expectorate samples from deep in the chest and spit them into a separate sample cup. Spirometry will be assessed after each saline level to ensure no lung function decrements. If the FEV1 decreases 10% or more from baseline, the subject will not be advanced to the next level of saline; however the same level may be repeated once. If the FEV1 drops 20% or more the procedure is stopped and, if necessary, 2 puffs of albuterol will be given. The physician may prescribe a second albuterol MDI treatment at his/her discretion.
10. Perform nasal epithelial cell biopsy for baseline: a non-invasive biopsy procedure will be performed to retrieve a small cluster of cells from each of the nasal cavities. For the biopsy procedure, the subject will be seated comfortably in a straight-backed chair or reclining on an exam table with the head titled as far back as possible while remaining comfortable. A short, sterile plastic sampling device called a curette will be inserted into one of the nasal cavities and the surface of the nasal cavity will be stroked several times for approximately 5 seconds in order to obtain a small cluster of cells. Only one side will be collected at this visit. The investigator, study physicians or other trained staff will collect these samples.
11. Introduce the subject to the chamber area by walking them through.
12. Subjects will be discharged with contact information for a study physician on call.

13. Psychological surveys: Once subjects have been qualified, they will be emailed a link to complete psychological surveys. These will be performed at home, and at the convenience of the subject, prior to the first exposure session.

Once the subject is deemed eligible, they will return for a baseline day to collect pre-exposure measurements. This visit must occur no less than 3 days and no more than 6 weeks after the screening visit. Subjects will be asked to withhold NSAIDs and antioxidant vitamins for 7 days prior, and to arrive fasting (water is acceptable). We will update any changes in medical history, including medication use, collect vital signs, and perform spirometry.

The following samples will be collected:

1. **Urine pregnancy test**: For all women, if more than 7 days have passed since the prior test.
2. **Epithelial lining fluid (ELF) collection**. This is done by having the subject spray normal saline into the nose, and then insert a small filter paper into each nostril. Nose clips are applied for 2 minutes, then the filter paper is removed. These samples are collected by study staff.
3. **Nasal lavage (NL)**: This is done by repetitive spraying of sterile normal saline irrigation solution into the nostril, followed by voluntary expelling of the fluid by the subject into a specimen collection cup. Both nostrils are sampled, these samples will be collected by study staff,
4. **Spirometry**: Standard spirometry to obtain FEV1 and FVC measurements will be done prior to sputum induction. This is done by having the subject exhale forcefully and completely into a mouth piece attached to a pneumotach. This will be repeated several times, and the procedure will be done following ATS/ERS standards.
5. **Flow-mediated brachial artery dilation (FMD)**: This measurement is made by imaging the brachial artery with an ultrasound device. The blood flow to the artery is occluded for 5 minutes, and the artery is again imaged when blood flow is resumed.
6. **Left ventricular strain:** Left ventricular contractility will be assessed by measuring global longitudinal strain using 2-dimensional speckle tracking echocardiography. Apical 4-chamber, 2-chamber, and long axis 2-dimensional ultrasound images of the heart will be acquired using a Philips EPIQ 7 ultrasound machine and stored for subsequent off-line analysis. Global longitudinal strain will be quantified using Philips QLAB advanced quantification software.

The subject will be discharged to home with contact information for a study physician who is on-call if the subject has any health concerns. The subject will return the next morning, and after health history is updated and vital signs collected, the following will take place:

1. **Heart rate variability (HRV)**: Standard ECG leads will be placed on the subject's chest, and connected to a monitor that records the heart rate and rhythm. The subject will be asked to lie supine in a quiet, darkened room for up to 30 minutes. The subject will do this before the exposure and immediately after the exposure. The analysis for this measurement is computer based.
2. **Spirometry:** Standard spirometry will be measured prior to the subject entering the exposure chamber.
3. **Ozone monitor**: Subjects will wear an experimental ozone monitor, which is on a bracelet. This is to validate the wearable monitor in an environment with known ozone concentration.
4. **Telemetry:** Standard 3 lead ECG monitoring will be done while the subject is in the exposure chamber.
5. **Symptom questionnaire:** Subjects will be asked to complete a symptom questionnaire prior to entering the chamber.

7. The subject will enter the chamber. The atmosphere will be determined randomly and will be either filtered clean air or ozone. The ozone concentration will be varied from 0.06 to 0.08 through the course of the exposure, and the exposure will last for 6.5 hours. The subjects will rest during the exposure with intermittent mild walking on the treadmill. Subjects will be provided with a low fat lunch to eat while they are in the chamber. HR will be monitored continually while the subject is in the chamber, and blood pressure will be measured hourly. A symptom questionnaire will be completed at the end of the exposure period. Immediately upon exiting the chamber, the subjects will undergo HRV for up to 30 minutes, followed by FMD and LV strain measurement. ELF and NL samples will be collected. Nasal epithelial cells (NEC) will be obtained from the opposing nostril from screening or exposure session 1.

Spirometry will be obtained prior to discharge, and at any point during the study if the subject experiences any distress or shortness of breath. Subjects will be discharged with contact information for a study physician on call.

Subjects will return 24 hours later. After updating the health history and collecting vital signs, the following measurements/samples will be collected:

1. Symptom questionnaire
2. HRV for up to 30 minutes
3. Venipuncture as collected at screening day
4. ELF
5. NL
6. Spirometry
7. Sputum induction

The subjects will be discharged with contact information for a study physician. They will be scheduled to return for a second session, with a minimum of 2 weeks and a

maximum of 6 weeks between exposures. The sessions will be identical except for the atmosphere in the chamber, which is randomized.

Baseline measurements provide safety information prior to each exposure. Given the level of the O3 and the procedures that subjects are asked to undergo, we do not anticipate any health concerns, but if they do arise, they will be immediate, therefore there are no scheduled visits beyond day 3 of the 2nd session. However, subjects will be encouraged to contact the study coordinator or physician at any time up to a month later if they have any concerns regarding their health or effects from the study.

4.1 Withdrawal Visit

If a subject withdraws from the study, any safety measures deemed necessary by the covering physician will be performed. This includes physical examination with vital signs, ECG measurement, or spirometry.

4.2 Unscheduled Visit

If a subject has an unexpected response to any study procedure, any safety measures deemed necessary by the covering physician will be performed. This includes physical examination with vital signs, ECG measurement, or spirometry.

4.3 Laboratory Procedures/Evaluations

4.3.1 Clinical Laboratory Evaluations

Blood will be sent to Labcorp, INC, for a complete blood count and chemistry values at screening.

4.3.2 Special Assays or Procedures

NLF will be collected and processed to obtain a cellular and cell-free fraction as described by us before, which will be analyzed for markers of inflammation and changes in immune responses. Cell-free nasal lavage fluid will be used to assess changes in markers of inflammation, host defense responses, and overall immune status. NLF cells will be used to assess changes in PMN influx as well as gene expression profiles associated with the exposure.

Superficial scrape biopsies will be obtained as described by us before and processed to yield both RNA and DNA. Total RNA will be analyzed for gene expression profiles associated with the exposures, while DNA samples will be used to determine epigenetic changes associated with the exposure.

Epithelial lining fluid (ELF) will be obtained by placing a strip of synthetic absorbent material (SAM) into the nostril, securing it with a nose clip, and removing it after about 2 minutes (you may want to copy language from our TCORS SOP here). ELF strips will be stored at -80oC until elution using PBS+BSA. ELF fluid will be used to assess changes in immune and inflammation mediators.

Sputum will be processed as previously published –in brief: plug material from sputum will be weighted and homogenized with DPBS (8x wt of selected plug material), centrifuged and supernatants recovered for proteomic assessment; sputum will be further homogenized with DTT (0.1%), filtered, centrifuged and the cell pellet recovered. The cell pellet will be re-suspended in an appropriate volume to a desired cell concentration ($1x10^6$ cells/ml) for cytospin slide generation; slides will be fixed and stained and read for differential leukocyte analysis. The remaining cells will be resuspended in Trizol reagent for RNA extraction and future mRNA/miRNA analysis using Nanostring technology.

Sputum: MesoScale Discovery to assay fluid phase proteins (cytokines); hemacytometry to assay total cell counts; NanoString technology to assay genes of interest; Flow cytometry: to assay functional cellular endpoints such as phagocytosis, intracellular oxidative burst, cell surface markers

In addition, we will genotype for SNPs of interest such as GSTM1 and other oxidant related genotypes; we will analyze mRNA expression for inflammation, immune and oxidant associated genes.

4.3.3 Reporting of SAEs and AEs to IRB

AEs and SAEs will be reported per UNC standard reporting procedures.

5 ETHICS/PROTECTION OF HUMAN SUBJECTS

5.1 Informed Consent Process

Informed consent is a process that is initiated prior to the individual agreeing to participate in the study and continues throughout study participation. Extensive discussion of risks and possible benefits of study participation will be provided to subjects and their families, if applicable. A consent form describing in detail the study procedures and risks will be given to the subject. Consent forms will be IRB-approved, and the subject is required to read and review the document or have the document read to him or her. The investigator or designee will explain the research study to the subject and answer any questions that may arise. The subject will sign the informed consent document prior to any study-related assessments or procedures. Subjects will be given the opportunity to discuss the study with their surrogates or think about it prior to agreeing to participate. They may withdraw consent at any time throughout the course of the study. A copy of the signed informed consent document will be given to subjects for their records. The rights and welfare of the subjects will be protected by emphasizing to them that the quality of their clinical care will not be adversely affected if they decline to participate in this study.

The consent process will be documented in the clinical or research record.

5.2 Subject Confidentiality

Subject confidentiality is strictly held in trust by the investigators, study staff, and their agents. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to any study information relating to subjects.

The study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party, however the subject may request copies of all clinical values, including lab results, ECG and spirometry.

6 DATA HANDLING AND RECORD KEEPING

Paper documents will be maintained by the PI or designee. All data will be entered into a REDCap database.

This study will comply with the *NIH Public Access Policy*, which ensures that the public has access to the published results of NIH funded research. It requires scientists to submit final peer-reviewed journal manuscripts that arise from NIH funds to the digital archive PubMed Central upon acceptance for publication.

The International Committee of Medical Journal Editors (ICMJE) member journals have adopted a clinical trials registration policy as a condition for publication. The ICMJE defines a clinical trial as any research project that prospectively assigns human subjects to intervention or concurrent comparison or control groups to study the cause-and-effect relationship between a medical intervention and a health outcome. Medical interventions include drugs, surgical procedures, devices, behavioral treatments, process-of-care changes, and the like. Health outcomes include any biomedical or health-related measures obtained in patients or participants, including pharmacokinetic measures and adverse events. The ICMJE policy requires that all clinical trials be registered in a public trials registry such as *ClinicalTrials.gov*, which is sponsored by the National Library of Medicine. Other biomedical journals are considering adopting similar policies. For interventional clinical trials performed under NIDCR grants and cooperative agreements, it is the grantee's responsibility to register the trial in an acceptable registry, so the research results may be considered for publication in ICMJE member journals. The ICMJE does not review specific studies to determine whether registration is necessary; instead, the committee recommends that researchers who have questions about the need to register err on the side of registration or consult the editorial office of the journal in which they wish to publish.

7 DATA ANALYSIS

Aim 1. Characterize ozone induced changes in nasal PMN counts.

This is a randomized, double blind, crossover pilot study, studying the changes in cells of nasal lavage (neutrophilla) after low-level ozone exposure. First, descriptive analysis will be conducted to examine potential ozone exposure effect and period effect. Second, two sample tests such as t-test and Mann Whitney test will be used to compare changes in response in clean air and ozone exposure groups. In general, carry-over effect is first tested. In the absence of significant carry-over effect, pooled data from both periods can be used to estimate the ozone exposure effect. Otherwise, only period 1 data can be used. We will report effect estimates, standard deviation as well as confidence intervals for ozone exposure effect and potential period effect. Finally, a random effects model will be fitted to assess the ozone exposure effect. Confidence intervals will be reported.

Aim 2. Characterize ozone induced changes in inflammatory cytokines in nasal epithelian cell (NEC) biopsy and nasal epithelial lining fluid ELF

The analysis plan is the same as in Aim 1.

Aim 3. Characterize ozone induced changes in FMD, HRV, LVS, Brachial artery ultrasound, ambulatory ECG measures, & other cardiovascular measures.

The analysis plan is the same as in Aim 1.

Aim 4. Characterize ozone induced changes in spirometric measures

The analysis plan is the same as in Aim 1.

Aim 5. Investigate the performance of a wearable monitor for ambient ozone measurement.

There are 2 current devices which will be used (descriptions and photos attached. The subject may wear one or both of these devices. The purpose of these devices at this time is strictly to assess the measured values obtained by the devices compared to the known O3 atmosphere. However, as these devices are prototypes there are two important questions. The

first is will the sensors technically perform appropriately thought the exposure while being worn by volunteers. In essence, the goal of this assessment is to simply see fi the devices work at all, and to identify specific technical modifications which may need to be made. The second goal is to determine if the readings of functioning wearable sensors correlate well with the readings of the standard ozone monitors present in the exposure chamber (that is to demonstrate that they are accurate). All of the ozone sensors are non-invasive. **These are exploratory endpoints**

In case of potential missing data, we will first carefully investigate the pattern of missing data and determine if it is informative missing. In case the missing is heavy, we will explore statistical methods such as multiple imputation to handle the missing data issue (Little and Rubin, 2014).

The pilot design and sample size is motivated by our earlier similar study (Peden et al, 1995). The goal of this pilot study is to determine whether low-level ozone affects a) nasal epithelial inflammation, and b) exploratory cardiovascular endpoints such as heart rate variability. If we find that cardiovascular endpoints are affected by low-level ozone in this small group, further studies will explore these, and perhaps other, cardiovascular markers and outcomes. The aim of these future studies will be to further characterize the cardiovascular effects of low-level ozone exposure.

The sample size for this study is determined on our primary endpoint outlined in Aim 1. The other aims are all secondary analysis using the same sample collected in Aim 1. We will not collect additional subjects for Aims 2-5. In previous literature, it is found that higher-level shorter duration ozone exposure caused a change in neutrophilia of 67 with a standard deviation of 31, while the change is 31 with a standard deviation of 17 in the clean-air group (Peden et al, 1995). The aimed effect size is 36. If we assume the significance level is at 0.05, a sample size of 13 subjects is adequate to ensure 90% power of detecting the effect. Approximately half of the subjects will receive air-ozone sequence, and another half will receive ozone-air sequence. Using a significance level of 0.05, the table below shows the sample size under various potential correlation coefficient. The calculation is based on a two-sided paired t test.

Effect size	Correlation coefficient	Power	N
36	0	0.8	10
	0	0.9	13
	0.3	0.8	8
	0.3	0.9	10
	0.5	0.8	7
	0.5	0.9	9

Note: N means that N/2 subjects receive ozone-air sequence, and another N/2 subjects receive air-ozone sequence.

We anticipate the SD for the proposed study should behave similarly to the previous study. Nevertheless, we inflated our sample size to account for the situation that the true SD is larger than we assumed - our proposed sample size n=15 is larger than the real sample sizes for achieving 80% power, which is n=8 for correlation coefficient 0.3, and n=10 for corr. coeff at 0, respectively.

The statistical analyses will be conducted under the supervision of Dr. Haibo Zhou (Professor of Biostatistics and Director of the CEMALB Biostatistics Core).

Reference:

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