Study Protocol

Carbon Dioxide (CO_2): A Pilot Study of a Hypothesized Mechanism to Explain Cognitive Impairment

NCT 05292378

November 18, 2024

INTERVENTIONAL
RESEARCH PROTOCOL TEMPLATE
(HRP-503a)

STUDY INFORMATION

- **Title of Project:**
  Carbon Dioxide: A Pilot Study of a Hypothesized Mechanism to Explain Cognitive Impairment

- **Principal Investigator Name**
  Howard M. Kipen, MD, MPH

- **Principal Investigator Div. & Dept.**
  EOHSI/Environmental and Occupational Health
  Rutgers School of Public Health

- **Principal Investigator Contact Info:**
  Kipen@eohsi.rutgers.edu
  EOHSI Room 208
  170 Frelinghuysen Road
  Piscataway, New Jersey 08854
  848-445-6091

- **Protocol Version and Date:**
  V10, 09.30.2024

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1.0 Research Design

1.1 Purpose/Specific Aims

The purpose of the study is to provide a mechanistic basis for the experimental phenomenon of CO_2-induced cognitive impairments in humans. Our highly novel focus is on biochemical changes caused by CO_2 through its interactions with reactive oxygen and nitrogen species (RONS). We speculate that elevated levels of CO_2 alter the actions of RONS within cells and that this leads to changes in their function.

A. Objectives

a. Use the Strategic Management Simulations (SMS) test, the Stroop Color-Word Test (SCWT), and the Wisconsin Card Sorting Test (WCST) to analyze the effects of CO_2 on cognitive function in healthy volunteers.

b. Determine whether CO_2 inhalation results in nitrosative and nitrative modification of target biomolecules in polymorphonuclear leukocytes (PMNs) and whether this correlates with PMN activation and oxidative stress.

c. Determine whether CO_2 inhalation results in cerebral vascular leak and whether this correlates with PMN activation and oxidative stress.

d. In Phase 3 - determine whether CO_2 inhalation results in cerebral vascular changes in vessel diameter and blood flow and whether this correlates with PMN activation and oxidative stress. In this pilot study our goal is to demonstrate the feasibility of using a novel noninvasive measure conjunctival these vascular changes to correlate with data we already collect in a, b, and c above.

B. Hypotheses / Research Question(s)

We hypothesize that exposure of humans to inhaled CO_2 at common indoor levels will lead to cognitive decrements due to CO_2-mediated nitrosation and nitration of intracellular biomolecules in PMNs, which cause cellular activation and oxidative stress.

1.2 Research Significance

Carbon dioxide (CO_2) is expired by people at a concentration of 40,000 ppm and is ubiquitous. Indoors, CO_2 accumulates in proportion to the number of individuals in a room or enclosed space, making indoor levels substantially greater than outdoor levels. CO_2 itself is generally considered non-toxic at levels up to 5,000 ppm, the federal standard for workplaces set by OSHA (U.S. Department of Labor). Based on this, guidelines for the International Space Station and U.S. submarines are to maintain CO_2 concentrations <5,000 ppm (National Research Council 2007). Levels up to 2,000 ppm are commonly measured in schools, businesses and homes, and levels up to 4,500 ppm are detectable in cars (Hudda and Fruin 2018) or inside the face masks of health care workers' N95 respirators (Roberge, Coca et al. 2010). CO_2 levels below 1,000 ppm are widely regarded as acceptable (U.S. EPA , Roberge, Coca et al. 2010, Allen, MacNaughton et al. 2019) despite the fact that indoor values in excess of 1,000 ppm are strongly correlated with non-specific symptoms of sick building syndrome, as well as poor school performance (Seppanen, Fisk et al. 1999, Fisk 2017). High CO_2 is commonly regarded only as an indicator of inadequate ventilation rather than a threat to health (National Research Council 2007).

Since 2012 four groups have published ten papers using varying experimental designs to explore acute (~2 hr) effects of real-world CO_2 concentrations (600-2,500 ppm) on cognitive performance of human subjects (Roberge, Coca et al. 2010, Kajtár and Herczeg 2012, Satish, Mendell et al. 2012, Allen, MacNaughton et al. 2016, Zhang, Wargocki et al. 2016, Liu, Zhong et al. 2017, Zhang, Wargocki et al. 2017, Rodeheffer, Chabal et al. 2018, Allen, MacNaughton et al. 2019). Four of the studies reported an association between CO_2 and decreased cognitive performance (Kajtár and Herczeg 2012, Satish, Mendell et al. 2012, Allen, MacNaughton et al. 2016, Allen, MacNaughton et al. 2019). All of these were

rigorous (within subject cross-over, double blind) studies and two demonstrated concentration-dependent impairment using a highly sensitive, validated measure of cognitive function, the Strategic Management Simulations (SMS) (Satish, Mendell et al. 2012, Allen, MacNaughton et al. 2016, Azuma, Kagi et al. 2018). A third rigorous study was performed more realistically, and provocatively, with professional airline pilots in a flight simulator (Allen, MacNaughton et al. 2019). Conversely, the null or inconclusive studies used less demanding traditional neuropsychological tests, non-within subject designs, and/or impure CO_2 (Roberge, Coca et al. 2010, Zhang, Wargocki et al. 2016, Liu, Zhong et al. 2017, Zhang, Wargocki et al. 2017, Rodeheffer, Chabal et al. 2018).

Without a fundamental mechanism, one cannot properly assess the harm that may follow CO_2 exposure. Our proposed studies are focused on elucidating mechanisms at both the physiological and biochemical level that underlie cognitive impairments associated with CO_2 exposure in humans. These mechanistic human studies are highly significant as there are major implications to systemically substandard performance at school, on aircraft, and in business. We intend to move the science beyond observation so that this important public health concern can be addressed both scientifically and at a regulatory level.

Chemically, CO_2 lacks direct toxic reactivity. However, due to the dielectrics of the molecule, the central carbon is susceptible to nucleophiles such as superoxide anion, nitric oxide and peroxynitrite, generating caged radical intermediates. The reaction of CO_2 with peroxynitrite generates a carbonate and nitrogen dioxide coordinated intermediate (Pryor, Lemercier et al. 1997, Zhang, Squadrito et al. 1997) that favors nitrosation and nitration of targets over simple oxidation of targets such as glutathione (Zhang, Squadrito et al. 1997, Squadrito and Pryor 1998, Uppu, Lemercier et al. 1998). This can have a major impact on signal transduction pathways and cellular responses (Gow, Farkouh et al. 2004). This is supported by findings that treatment of isolated human PMNs with CO_2, at realistic levels, enhances cellular activation as measured by an increased oxidative burst and the release of IL1βcontaining microparticles (Thom, Bhopale et al. 2017). Similar effects on PMNs were observed in rodents following CO_2 inhalation (2,000-4,000 ppm), a response correlated with brain vascular leak (Thom, Bhopale et al. 2017). Importantly, vascular damage in the brain was abrogated by pre-treatment of mice with IL1β antibodies or an IL1β receptor antagonist. Moreover, when naïve mice were injected with IL1β containing microparticles derived from CO_2 treated PMNs, vascular damage was recapitulated, while destruction of microparticles abrogated the vascular injury with no change in PMN activation. These data are in accord with reports that IL1β and other members of the IL1 family (e.g., IL18, IL33) induce vascular leak (Fahey and Doyle 2019). These findings suggest a causal link between PMN activation by CO_2, microparticle/IL1β generation, changes in cerebral vessels and blood flow, and cognitive impairments, consistent with the relationship between inflammation and cognition (Shields, Moons et al. 2017, Jacobson, Kler et al. 2019), which we propose to investigate in humans. Inclusion of functional magnetic resonance imaging (fMRI) will provide visualization and enhanced characterization of the cerebral vascular leakage predicted by our hypothesized mechanism of CO_2-mediated cognitive toxicity. In Phase 3, the addition of computer-assisted intravital microscopy (CAIM) will provide noninvasive visualization of a representative part of the cerebral vasculature, giving further insight into the hypothesized mechanism. Positive results from our high risk-high reward translational studies will provide important mechanistic data on CO_2 and impaired cognitive function that may lead to consideration of revisions to current acceptable CO_2 exposure levels.

We contend that CO_2 mediates changes in the reactivity of reactive nitrogen and oxygen species (RONS) and that this increases PMN activation. Therefore, baseline oxidative stress defenses in the subjects may affect the response to CO_2. In this context, the status of glutathione S-transferase, (GSTM1*)*, a phase II antioxidant enzyme, may be critical to responsiveness. The *GSTM1* null genotype, lacking GSTM1 protein, is associated with increased risk of adverse health effects due to exposure to air pollutants as we and others have shown (Schwartz, Park et al. 2005, Lee, Kim et al. 2022). *GSTM1* has been reported to be null in approximately 50% of Caucasians, African American, and Asian populations, consistent with the diverse recruiting pool of subjects in New Jersey and providing greater

power for statistical analysis than other GST's with lower null frequencies (Mo, Gao et al. 2009). Based on these frequencies in our recruitment pool, we believe, *GSTM1* genotype represents the best variable to examine a hypothesis that antioxidant status and thus oxidative stress are mechanistically important for understanding how CO2 could affect cognition though inflammation.

1.3 Research Design and Methods

The study is a crossover trial. Subjects and outcome assessors will be blinded to the CO_2 exposure level. The study will be done in two phases. The main endpoint for Phase 1 will be markers of PMN activation and oxidative stress in blood samples. In Phase 2, fMRI will be added to the study procedures, and fMRI evidence of cerebral vascular leak will be added as an endpoint. In Phase 3, we are no longer using fMRI, but we have added computer-assisted intravital microscopy (CAIM), and evidence of changes in cerebral vessel diameter and blood flow will be added as endpoints

Research Procedures – Phase 1, 2, and 3

Subjects will participate in two 2.5-hour exposure sessions in the Controlled Environment Facility (CEF) located in the Rutgers Environmental and Occupational Health Sciences Institute (EOHSI) building. Concentrations of CO_2 during the exposure session will be either 600 ppm (control) or 2500 ppm (exposure). Concentrations in the CEF will be continuously monitored during the exposure, and while keeping ventilation constant, the flow rate will be adjusted to maintain CO_2 levels. The order of the exposure sessions will be randomized and the exposure sessions will be at least one week apart. Up to four subjects will participate in each exposure session.

During the exposure session, each subject will have their own desk and laptop computer for the neurobehavioral testing and be visually and audibly (via headphones) isolated from the other subject. Since face masks have been shown to increase the concentration of carbon dioxide (Rhee, Lindquist et al. 2021), subjects will be asked to remove face masks while they are in the chamber. Subjects will be acclimatized in the CEF for 60 minutes with the selected CO_2 exposure concentration already stabilized in the chamber atmosphere before they enter. During the last 30 minutes of acclimatization, subjects will be instructed on the neurobehavioral Strategic Management Simulations (SMS) test. Otherwise, they may read/study quietly without music or phone calls. The neurobehavioral testing will consist of the SMS test (about 1.25 hours) and the SCWT (Phase 1 – about 5 minutes) or the WCST (Phase 2 – about 15 minutes).

Height and weight will be measured at the first study visit.

Subject symptoms and air quality perceptions will be assessed via questionnaires before entering the chamber and following each exposure session.

Venous blood will be collected from subjects at three time points at each visit: immediately before entering the chamber (30 mL), immediately after the exposure (10 mL), and approximately four hours after exposure ended (30 mL).

In Phase 2 only, after each exposure, subjects will undergo a 60-minute fMRI scan during which they will participate in Multi-Source Interference Task (MSIT).

A. In Phase 3 only, after each exposure, subjects will have up to five minutes of video recorded of the white of the eye using computer-assisted intravital microscopy (CAIM) CAIM is a non-invasive technology that quantifies microcirculation abnormalities. **Data Points**

SMS, SCWT, and WCST scores will be collected at each exposure. Biomarkers of PMN activation and oxidative stress will be measured before and after each exposure. In Phase 2, the presence of cerebral vascular leak will be assessed by fMRI. In Phase 3, microcirculation abnormalities will be recorded using CAIM and later analyzed in the lab of Dr. Joshua Miller.

B. Study Duration

The study is expected to take two years. Each subject will participate in 2 study visits for a total time of approximately 16 hours. In Phase 3, each subject will participate in 2 study visits, for a total time of approximately 8 hours.

C. Endpoints - NA

1.4 Preliminary Data

Indoor CO_2 levels were recently measured in 29 homes in New Jersey (Pro2020001323; A Randomized Crossover Trial of Portable Air Cleaners to Reduce Coronavirus Exposures at Home). The average CO_2 concentration was 825 ppm with three homes having average levels exceeding 1500 ppm. Two homes were observed to have sustained periods (>1 hour) of exposure to levels exceeding 2500 ppm.

1.5 Sample Size Justification

Twelve subjects will be enrolled in phases 1 and 2 of the study. The sample size was based on finding a difference in in PMN activation and oxidative stress. Using published data (Thom, Bhopale et al. 2017) and assuming that ex vivo experiments are conducted on repeated samples from the same individuals and an intra-subject correlation of 0.7, a sample size of 6 has over 95% power to detect an effect. An additional up to ten subjects will be enrolled in phase 3 of the study. The purpose of this phase 3 portion of the study is to demonstrate the feasibility of using the CAIM technique as an outcome variable in our studies. Significant results of the cognitive testing and PMN activation are already reported (Lu et al., ink press). We do not anticipate statistically significant changes in this pilot investigation

1.6 Study Variables

A. Independent Variables, Interventions, or Predictor Variables

The independent variable is the CO_2 concentration. Two levels of CO_2 will be used during the exposure sessions: 600 ppm (control) and 2500 ppm (exposure).

We will also assess the GSTM1 genotype (present/absent) of the subject. In Phase 3 we will not assess the GSTM1 genotype of the subject.

B. Dependent Variables or Outcome Measures

The primary outcome measures are cognitive function assessed by SMS, SCWT, and WCST, biological markers of PMN activation and oxidative stress, and cerebral vascular leak assessed by fMRI with a 3D diffusion-prepared arterial spin labeling (ASL) perfusion sequence.

In Phase 3 of the study changes assessed in the bulbar conjunctiva as measured by the CAIM technique will be added as a primary outcome measure.

SMS testing assesses up to nine primary factors of cognitive function: basic activity level, applied activity, focused activity, task orientation, initiative, information search, information usage, breadth of approach, and basic strategy.

SCWT responses will assess error rates and response times. WCST results will be scored by the number of errors, perseverative responses, and completed categories.

Blood samples will be examined for oxidative burst, mitochondrial energy metabolism, and NLRP3 inflammasome activity as reflected by activated caspase-1 and expression of markers of inflammasome activation (e.g., IL1β, IL18, MYD88). We will also assess microparticles (e.g., extracellular vesicles) in peripheral blood samples, their origin (e.g., PMNs), and their vasoactive cargo (e.g., IL1β, IL18, IL33) (4), as well as serum levels of these cytokines. A key component of our studies will be to examine changes in nitrosation and nitration of potential proteins (e.g., cytochrome c oxidase, aconitase, GAPDH, NFκB, PPARγ, PKCs) and lipids (e.g., nitro-oleic and nitro-linoleic acids) within PMNs. In Phase 1, RNA sequencing will be done to assess additional markers of PMN activation (at the pre-exposure and 4-hour post-exposure time points).

1.7 Drugs/Devices/Biologics - NA

1.8 Specimen Collection

A. <u>Primary</u> Specimen Collection

- **Types of Specimens**: Blood will be collected at 3 time points for each exposure session (70 mL total/exposure): pre-exposure (30 mL), post-exposure (10 mL), and 4 hours post-exposure (30 mL).
- **Annotation**: Blood samples will be annotated with the study ID, the exposure session number, and the time point (pre and post). No identifiers will be used on the specimen containers.
- **Transport**: Specimens collected pre-exposure and 4 hours post-exposure will be carried from EOHSI to Dr. Gow's lab in the Ernest Mario School of Pharmacy by a research assistant. In Phase 1 only, after processing, an aliquot of RNA isolated from the PMNs will be shipped to Baylor College of Medicine for RNA sequencing on a fee-for-service basis. Shipping will be done by a laboratory assistant with current IATA training.
- **Processing**: Laboratory assistants in Dr. Gow's lab will process the blood collected pre-exposure and 4 hours post-exposure. Specimens collected immediately post-exposure will be processed by research assistants at EOHSI, Room 145.
- **Storage**: Processed specimens will be stored in -80°C freezers in EOHSI Room 232 until analysis in Dr. Gow's lab or, in Phase 1 only, shipping to Baylor College of Medicine. Access to the room is controlled by key card.
- **Disposition**: Specimens sent to Baylor University will be destroyed after all data have been reviewed and approved. Remaining specimens in Dr. Gow's lab will be stored indefinitely for use only by the study investigators. The link between the study ID and the subject identity will be destroyed when the study is closed.

B. Secondary Specimen Collection - NA

1.9 Data Collection

A. Primary Data Collection

- **Location**: The SMS, SCWT, and WCST data collection will take place in the controlled exposure facility (CEF) of Rutgers Environmental and Occupational Health Sciences Institute (EOHSI). In Phase 2, the fMRI will be done at the Center for Advanced Human Brain Imaging, 119 Staged Research Building, Piscataway, NJ. In Phase 3, the SMS data collection will take place in the CEF at EOHSI, and digitized conjunctival images taken using the CAIM technique will take place in the EOHSI Clinical Center subject room (room 145).
- **Process of Data Collection**: An online SMS test will be administered; a research assistant, trained in test administration, will provide instruction and be available to answer any questions during testing. Online versions of the SCWT and WCST will also be administered. The fMRI will follow a standard sequence (see below) to assess vascular leak.
- **Timing and Frequency**: The tests will be administered once during each exposure session. The SMS test will take 75 minutes each time, and the SCWT will take 5 minutes. In Phase 2, the WCST will take 15 minutes. The fMRI will take approximately 1 hour.
- **Procedures for Audio/Visual Recording**: NA
- **Study Instruments**: The neurobehavioral SMS test is a highly sensitive measurement of higher order decision-making that has been used successfully to assess cognitive effects of allergy medication and alcohol, as well as exposure to CO_2 (Streufert, DePadova et al. 1988, Streufert, Pogash et al. 1994, Breuer and Satish 2003, Satish, Streufert et al. 2004, Satish, Mendell et al. 2012, Azuma, Kagi et al. 2018, Allen, MacNaughton et al. 2019). Another advantage of SMS is that parallel forms are available which can be used to control for practice effects from repeated administrations. Importantly, numerous publications support SMS test retest reliability (r = 0.72 to 0.94) for parallel forms of the test (Streufert, DePadova et al. 1988, Streufert, Pogash et al. 1994, Breuer and Streufert 1995, Satish, Streufert et al. 2004, Satish, Mendell et al. 2012, Allen, MacNaughton et al. 2016), as well as predictive validity of SMS for real world situations such as surgical residents' decision making (Satish, Streufert et al. 2001). Once the task commences, messages appear on the computer with instructions and participants are asked to respond with a drop-down menu of possible decisions. Although the same amount of information is presented

at fixed time points to each participant during the session, they are free to make decisions at any time, similar to real world situations. Scores are generated by the computer, based on previous factor analysis to reflect the participant's performance on the following measures: Activity – number of actions taken; Speed – speed of response to information; Responsiveness: orientation to task; Initiative: actions with creativity; Information: activities to seek information; Emergency: actions in response to emergency events; Breadth: use of multiple approaches for problem solving; Planning: plans for future action; Strategy: multiple interactive activities toward complex goals (Streufert, DePadova et al. 1988, Satish, Mendell et al. 2012).

The SCWT is a commonly used measure of executive function (Scarpina and Tagini 2017). The test consists of three conditions (naming the color of dots, of neutral words, and of the color of words printed in incongruent colors). Each condition consists of 24 items. Error rates and response times are evaluated.

The WCST is another commonly used measure of executive function and cognitive flexibility (Miles, Howlett et al. 2021). We will use the computerized 64-item version.

For fMRI, a 3D diffusion-prepared pseudo-continuous arterial spin labeling (ASL) perfusion sequence allows mapping of water exchange across the blood-brain barrier (BBB) without contrast, facilitating identification of vascular leak (Shao et al., 2018;). After removal of any metal, each participant will be positioned in a Siemens 3T Siemens Prisma with a 64-channel head coil. After a 1-minute scout navigator scan is collected for positioning of scans, a 5-minute structural T1-weighted scan will be collected. This will be followed by an approximately 10-minute scan to determine BBB diffusion and blood oxygenation level dependent (BOLD) imaging of the Multi-Source Interference Task (MSIT). The MSIT is a validated fMRI task that reliably and robustly activates a subject's cingulo-frontal-parietal cognitive/attention network (CFP network) to further assess cognition (Bush and Shin, 2006). An additional 14 minutes of resting-state fMRI to measure functional connectivity will be collected at the end of the scan. During all scans, a trained MRI technician will observe the participant through a large window and communicate by two-way microphone.

- **Ethnographic Studies, Interviews, Or Observation**: NA
- **Subject Identifiers**: Tests will be coded by subject ID and exposure session number.

B. Secondary Data Collection - NA

1.10 Timetable/Schedule of Events - NA

2.0 Project Management

2.1 Research Staff and Qualifications

Howard Kipen, MD, MPH, Professor, Environmental and Occupational Health, Rutgers School of Public Health. Dr. Kipen specializes in controlled exposure-based experimental studies of individuals' responses to changes in air pollutants, such as we propose here. He has led multiple federally funded field studies such as those proposed here including a recently completed study with portable air cleaners.

Andrew Gow is a Professor of Pharmacology at Rutgers University. He has 30 years of experience in academic research and has held positions at Princeton University, Temple University, University of Pennsylvania, and Duke University. Dr. Gow's primary research focus is nitric oxide chemistry and he has published extensively on the mechanisms of cardiac hypertrophy, environmental effects on nitric oxide signaling, pulmonary function and mechanisms of disease, and the role of nitric oxide in controlling oxygen delivery and inflammation. Dr. Gow's work has led to the understanding of S-nitrosothiols as a major factor in controlling nitric oxide function and has yielded technologies related to a variety of pathologies including persistent pulmonary hypertension of the newborn, the regeneration of stored blood, cystic fibrosis, sickle cell anemia, and asthma. Dr. Gow holds a BSc (Biochemistry) from the University of Edinburgh, a MSc in Physical Education, and a PhD in Exercise

Physiology from Temple University. He has published over 120 papers and has been cited over 7,500 times with an *h*-index of 41. He has given invited lectures across the world, including Japan, Germany, Russia, and the UK. Dr. Gow is a chartered chemist and a member of the American Physiological Society, the American College of Sports Medicine, AAAS, ASPET, SOT, and ATS; as well as being a past president of the International Nitric Oxide Society. He is a former personal trainer and rugby player.

Nancy Fiedler, Professor, Department of Environmental and Occupational Health, Rutgers School of Public Health and Deputy Director of the Environmental and Occupational Health Sciences Institute. Dr. Fiedler is a clinical psychologist and routinely performs neuropsychological evaluations of workers and community members who report symptoms related to neurotoxicant exposures.

David Zald, PhD, Henry Rutgers Professor of Psychiatry and Director of the Center for Advanced Human Brain Imaging Research (CAHBIR). He received training as a neuropsychologist and neuroimaging researcher. He has been conducting functional neuroimaging research for over a quarter of a century.

Joshua Miller, PhD, Professor and Chair in the Department of Nutritional Sciences in the School of Environmental and Biological Sciences. His recent research primarily focuses on the relationships among vitamins (B12, folate, B6, and D), homocysteine, and neurodegenerative diseases, age-related cognitive decline, and cancer. He has extensive experience in the measurement of various biomarkers of B vitamin status in large-scale epidemiological studies. He is currently subcontracted co-PI on an NIH-funded study of B12 status, cognitive decline, and incident dementia (AG059011 – PF Jacques, lead PI) and a subcontracted co-investigator on an NIH-funded intervention trial of vitamin D supplements to slow cognitive decline in older adults (AG051618 – J Olichney, PI). With respect to the present proposal, he has co-authored papers utilizing computer-assisted intravital microscopy (CAIM) to assess the peripheral microvasculature in sickle cell disease patients, and now endeavors to expand this novel technology to the study of early nutritional and vascular biomarkers of brain injury and age-related cognitive decline. His lab has purchased and operates a CAIM which will be transported to EOHSI for this pilot study.

Gina Roslan, BS**,** Graduate student in the Department of Nutritional Sciences. She has worked with computer-assisted intravital microscopy (CAIM) in Dr. Miller's laboratory on a previous project and is trained in its use. Along with Dr. Miller, she will assist in the training of research staff on the operation of the CAIM.

Kathy Black, PhD, MPH, Senior Research Associate, Rutgers Environmental and Occupational Health Sciences Institute. Dr. Black will act as the study coordinator and will be responsible for the training and supervision of the study research assistants. Dr. Black has over 20 years of experience coordinating environmental health studies, including Dr. Kipen's recent study using air cleaners in homes.

2.2 Research Staff Training

Dr. Kipen and Dr. Black will review all study procedures with the personnel prior to any enrollment. Dr. Fiedler will train the study staff to administer both the SMS and VST. Dr. Black will directly supervise the study research assistants and review the study records to ensure that consent has been properly documented and all data recorded. Dr. Kipen will meet weekly with the study team to discuss study progress. In Phase 2, Dr. Zald, director of the CAHBIR, will oversee the MRI protocol. The person running the MRI scanner must have received certified training to run the MRI scanner and must have Level 3 safety training consistent with the operating procedures of the CAHBIR. Any assistants on the project must have at least Level 2 safety training. The person reviewing the screening form must have at least Level 2 training and is required to notify the person running the scanner that the review sheet had no items of note or that the sheet requires secondary review if any questions on the review sheet

were scored in the affirmative. Dr. Zald will oversee the overall study, whereas the person operating the scanner directly oversees the MRI data collection during the session itself. In Phase 3, Dr. Miller and Gina Roslan will train study staff in the procedures for CAIM.

2.3 Other Resources

Laboratory: Dr. Gow occupies approximately 1500 sq. ft. of newly renovated laboratory space in the Ernest Mario School of Pharmacy building (room 009) on the Busch Campus of Rutgers University; this laboratory, along with 2000 sq ft of adjacent laboratories (room 001) are available to perform laboratory studies outlined in this application. The School of Pharmacy is connected by an enclosed walkway to the Rutgers Environmental and Occupational Health Sciences Institute (EOHSI), which houses the laboratories, equipment and facilities for performing human exposure studies (see Clinical) below). Dr. Gow's laboratories are fully equipped with all of the basic and specialized equipment needed for the proposed, including analytical balances, pH meters, refrigerators, freezers, low and high speed centrifuges, water baths, gel electrophoresis equipment and power supplies, perfusion pumps, histology stations, a thermocycler, a hybridization oven, spectrophotometer, fluorescence plate reader, Shandon cytocentrifuge, microcentrifuge, and microwave, and an NOA-280i nitric oxide analyzer. Dr. Gow's laboratories have dedicated for tissue culture facilities which include laminar flow hoods (4), incubators (4), cell culture and fluorescent microscopes with digital imaging. Also available for use in the laboratories are desk top computers and printers for data analysis and processing. Major shared equipment in the School of Pharmacy and the EOHSI building available for this project include ultracentrifuges, freezers, scintillation and gamma counters and gel scanners. Common cold rooms and dark rooms are also available for use.

Controlled Environmental Facility (CEF): Human CO_2exposures will be conducted in the EOHSI CEF under the direction of Dr. Laurent. The CEF consists of three major components: 1) the exposure chamber; 2) the air pollutant generation system; and 3) the control systems. The CEF is a large stainless steel room (7.3 ft. high x 13.5 ft. wide x 9 ft. deep; volume=887 cubic feet) in which the air flow, temperature and humidity can be varied and well controlled. The airflow rate through the CEF can vary from 100 to 700 ft3/min. The CEF operating temperature range is from 55° to 80° ± 1° F. The relative humidity range is 10% to 80% ± 2%. A divider can separate the chamber into two work station compartments, so that two subjects can be exposed simultaneously. For the 600 ppm exposure, ambient air that supplies the exposure chamber will be treated using a series of processes, which include cooling/heating, humidification/dehumidification, and filtration through a carbon filter, a KMnO4 purifier, and HEPA filters. The clean air enters the CEF through two diffusers located in the ceiling and exits through the perforated stainless steel floor without recirculation. Eight small brushless fans (to prevent unwanted particle generation from brush degradation) are used to ensure that the air is well mixed at the edges and throughout the stainless steel chamber. The temperature and relative humidity will be maintained at 72±1ºF and 45±2%, respectively, during each exposure session. While the CEF can be operated under either a positive or negative pressure of 0.1 inches of water, a negative pressure will be used to avoid CO_2 releases from the CEF to the surrounding rooms. A high purity (>99.999) CO_2 gas cylinder will be used to generate the desired CO_2 level within the CEF. The tank will be directly connected to a port leading to the inlet air for the CEF prior to the baffle system that is designed to mix the target agent(s) with the intake air before entering the CEF. The CO_2 concentration will be continuously monitored by the CEF operator to be within 10% of the target value and the tank flow rate will be adjusted using a fine needle valve to maintain that level throughout the exposure scenario. Since people are a source of CO_2, adjustments to the gas flow will be made as needed across the exposure session based on the number of people in the CEF and their breathing levels. In addition, there is a one-way mirror and a microphone so the participants can be monitored to verify that they are not feeling any distress. If the CO_2 level is not maintained or distress is indicated, then the experiment is stopped with the subjects brought out of the exposure session and evaluated by a physician from EOHSI clinic in the same building. Subjects enter the chamber through an airlock with two doors, one opening to the

outer room and the other opening into the chamber. Each door has a sensor which, when the door is opened, activates a sliding bolt to the other door, preventing it from being opened. When the door is closed, the sensor deactivates the bolt and either door can then be opened. This prevents both doors from being opened simultaneously and thus prevents air exchange between the outer room and the chamber. There is a 4-foot wide emergency exit in the front of the chamber leading directly to the outer room. The latches on the doors are designed to prevent anyone from being locked in the room. Other safety features include a smoke detector, a sprinkler system and battery operated emergency lighting. Researchers can view subjects participating in human exposure studies through a 4 x 6 ft. one-way mirror in the front of the CEF. There is a system for continuous electrocardiograph recording and monitoring of subjects during exposure. There is a lavatory in the chamber for extended exposure studies.

The Clinical Center for Environmental and Occupational Health: The EOHSI Clinical Center is the leading facility in NJ for workplace health. Directed by Dr. Kipen, there are 8 nurses and 4 FTE physicians in the EOHSI Clinical Center. The Clinical Center provides a wide variety of services including diagnosis, treatment and rehabilitation related to workplace exposures and injuries, clinical toxicology consultation, management of medical surveillance programs, biomonitoring for toxins, and evaluation of indoor air quality. The EOHSI Clinical Center also provides medical monitoring and treatment for ~2,500 WTC rescue, recovery and clean-up workers as part of a consortium of eight NIOSH designated Centers of Excellence. The clinical staff occupies space two floors below the CEF in the EOHSI building. Clinical Center staff will be on-call during exposures.

The Center for Advanced Human Brain Imaging: CAHBIR is equipped with a 3T Siemens Prisma with a 64-channel head coil. The center is equipped with a crash cart if a subject needs immediate intervention while at the scanner, and the MR technologist is trained in its use.

2.4 Research Sites

The study will take place at Rutgers-EOHSI (170 Frelinghuysen Road, Piscataway, NJ 08854). All data and specimen collection will take place at EOHSI including recruitment, CO2 exposure, neurobehavioral testing, and blood collection.
Specimens will be analyzed at Rutgers-EMSOP (160 Frelinghuysen Road, Piscataway, NJ 08854).
The fMRI will take place at the CAHBIR, 119 Staged Research Building, Piscataway, NJ.

3.0 Multi-Center Research - NA

4.0 Subject Considerations

4.1 Subject Selection and Enrollment Considerations

A. Method to Identify Potential Subjects

Subjects will be broadly recruited from the University and local communities. A screening questionnaire will be administered to determine eligibility.

B. Recruitment Details

Approved flyers will be posted around the University and local community. Study information will also be posted on the EOHSI website.
If interested, subjects will call or email the study coordinator or research assistants. A screening questionnaire will be administered to determine eligibility. The screening questionnaire will be reviewed by the study physician for approval prior to scheduling a study visit. In addition to the screening questionnaire, a pregnancy test will be given to all female participants prior to each exposure session. The short-term CO2 exposures used in this study are seen in everyday life, including schools and workplaces, and are not expected to cause clinically significant or persistent

health effects. Phase 2 participants will be given an additional screening questionnaire for fMRI compatibility to be completed before the MRI scan. No additional physical screening will be used. Pregnancy and current asthma have been included in the exclusion list out of an abundance of caution.

C. Subject Screening

- **Inclusion Criteria**

  Inclusion criteria include:
  - Age between 18 and 30 years (inclusive)
  - History of COVID-19 vaccination
  - Weigh at least 110 pounds
- **Exclusion Criteria**

  Exclusion criteria include:
  - Colorblindness
  - Inability to hear verbal instructions
  - Cardiovascular disease, including a history of stroke
  - Diabetes requiring the use of insulin
  - Pregnancy
  - Current asthma (an asthma attack within the past five years)
  - History of a severe anxiety or panic disorder
  - Medications for anxiety disorder
  - Ever experienced a panic attack
  - Medications which may affect cognition such as beta-blockers and CNS depressants
  - Respiratory symptoms in the previous 4 weeks
  - Use of sedating cold/allergy medications in the previous week
  - Use of marijuana in the previous week
  - Consumption of alcohol in the previous 24 hours

Additional exclusion criteria for Phase 2 participants are:

- History of head trauma or neurosurgery or neurological disorder
- Ferrous material implanted in or on the body, including surgical clips, bullets, electrical devices such as a pacemaker, or nonremovable ferrous jewelry (fillings in teeth and permanent retainers are permitted).
- Individuals with surgical pins or plates above the neck are excluded. Surgical pins or plates below the neck are exclusions, except when the material is fixed to bone, and considered acceptable by the *Reference Manual for Magnetic Resonance Safety. Implants and Devices, 2020 Edition*. Almost all recent orthopedic implants are made of materials that are not ferromagnetic and therefore are safe for scanning, and even though some screws are still made of ferromagnetic materials these are firmly screwed into bone. In cases where the material is unknown or deemed unsafe for scanning by the *Reference Manual for Magnetic Resonance Safety. Implants and Devices, 2020 Edition*, the participant will be excluded.
- History of eye injury involving metallic materials, shavings in eyes, or welding without a face mask
- Lead/iron tattoos
- Claustrophobia (history of significant anxiety in closed places).
- Back problem that would prevent the subject from laying still comfortably for up to 90 minutes.
- Deafness

Additional exclusion criteria for Phase 3 participants only:

- Contra-indications for CAIM
  - Inability to keep head still and eyes open during CAIM recordings
  - Current or recent contact lens use

D. Privacy Protections

Prospective subjects must respond to posted study information by contacting investigators. Individuals will not be approached for participation. Contact information collected during recruitment will be stored in the REDCap database, accessible only to the study team.

4.2 Obtaining Identifiable Information About Non-Subjects - NA

4.3 Number of Subjects

A. Total Number of Subjects

34 subjects will complete the study. To compensate for voluntary withdrawals, up to 44 subjects may be enrolled.

B. Total Number of Subjects If Multicenter Study - NA

C. Feasibility

Given the size of the University community, broad inclusion criteria, and the small number of subjects, we expect to be able to complete testing in a timely manner.

4.4 Consent Procedures

A. Consent Process

- **Location of Consent Process**
  Screening will take place over the phone. If the subject is on any medications, the list of medications will be reviewed by the study physician (Dr. Kipen) to determine eligibility. If eligible, an appointment will be made for a study visit. Subjects will be consented in the EOHSI Clinical Center (Room 145). The consent will be given to the study subject and the subject will be asked to review the consent prior to the study visit. At the start of the first exposure visit, the consent will be reviewed with the subject and signed by both the study participant and the investigator. After consent, the subject's height and weight will be collected and, if female, a pregnancy test will be administered.
- **Ongoing Consent**
  Study activities will be discussed with the subject at the start of each exposure visit. All questions will be answered.
- **Individual Roles for Researchers Involved in Consent**
  Screening and consenting may be done by the coordinator or a research assistant.
- **Consent Discussion Duration**
  The consent process will begin during screening when a brief explanation of the study is given to the subject. After screening, the consent form will be given to the subject to review prior to the exposure study visit. At a minimum, the subject will be given at least overnight to review the consent, but most appointments will be scheduled at least several days in advance. At the first exposure visit, the consent form will be reviewed with the subject and all questions will be answered before signing; this discussion is expected to take approximately 30 minutes.
- **Coercion or Undue Influence**
  Subjects are not approached individually, rather the study will be discussed only with subjects responding to a study flyer.
- **Subject Understanding**
  The key study elements will be specifically mentioned during the consent discussion and all questions will be answered.
- **Protecting Privacy**
  Consent discussions will take place in a private office prior to participation. No additional information about the subject will be collected until after consent is obtained.

B. Waiver or Alteration of Consent Process - NA

C. Documentation of Consent

- **Documenting Consent**
  The consent form will be stored in REDCap. Both the participant and study investigator will sign the consent form. A copy of the signed consent form will be emailed to the participant.
- **Waiver of Documentation of Consent (i.e., will not obtain subject's signature) - NA**

4.5 Special Consent Populations

A. Enrolling Minors-Subjects Who Are Not Yet Adults - NA

B. Enrolling Wards of the State - NA

C. Enrolling Non-English-Speaking Subjects - NA

D. Enrolling Adults Lacking Decision-Making Capacity (Surrogate Consent) - NA

E. Special Consent Considerations - NA

4.6 Economic Burden and/or Compensation for Subjects

A. Expenses - NA

B. Compensation/Incentives
Subjects will be paid $100 for each exposure visit (up to $200 total).

C Compensation Documentation
Compensation will be documented in the REDCap database. Additionally, subjects will be asked to sign a voucher. Copies of the voucher will be stored in a locked cabinet in a private office (Room 147) at EOHSI.

4.7 Risks of Harm/Potential for Benefits to Subjects

A. Description of Risks of Harm to Subjects

- **Reasonably Foreseeable Risks of Harm**

Carbon dioxide exposure: CO_2 itself is generally considered non-toxic at levels up to 5,000 ppm, the federal standard for workplaces set by OSHA ((U.S. Department of Labor). The CO_2 levels used in the exposures will not exceed those often indoors in conference rooms and schools; these levels have been used previously to assess changes in cognitive function with no adverse events. There are no known persistent sequelae to acute exposures in healthy individuals. Subjects may increase their respiratory rate during the high CO_2 exposure, but this is transient. Inhalation of CO_2 at approximately 35,000 ppm will reliably induce panic attack symptoms in those with a history of such attacks, but this is not reported at the order-of-magnitude lower concentrations that we will use.

Venipuncture: Slight pain, some bleeding, or bruising may occur when blood is drawn. A total of 100 ml of blood (50 ml per study visit) will be required for the planned assays.

Phase 3 only:
MRI Risks: There are no known health risks associated with the magnetic field produced by the 3.0T scanner in healthy adults. The FDA has indicated that they consider MR imaging on machines up to 4.0T to pose no risk. Exposure to high magnetic fields is associated with

primary or secondary risks in certain patient populations (e.g., patients with pacemakers), but all such patient populations are excluded. Subjects with aneurysm clips, neural stimulators, possible metal fragments in the eyes, cochlear implants, artificial cardiac valves, iron based facial tattoos, and body piercings that are not removable are excluded from participation. The only other risks associated with scanning are claustrophobia while in the magnet, physical discomfort from lying still in the magnet, and the loud sound of the magnet.

- **Incidental findings:** The MRI exam could reveal a neural abnormality. Learning of such an abnormality could cause psychological distress.
- **Risk of Harm from an Intervention on a Subject with an Existing Condition - NA**
  The study will not enroll subjects with existing conditions.
- **Other Foreseeable Risks of Harm**
  There is a risk of possible loss of confidentiality, but no sensitive information is collected in the study.
- **Observation and Sensitive Information - NA**

B. Procedures which Risk Harm to Embryo, Fetus, and/or Pregnant Subjects - NA

C. Risks of Harm to Non-Subjects - NA

D. Assessment of Social Behavior Considerations - NA

E. Minimizing Risks of Harm

- All subjects will be screened prior to scheduling, and the responses will be reviewed by the study physician PI prior to enrollment. Subjects with a diagnosis of anxiety disorder, history of panic attacks, or taking medication for anxiety disorder are excluded from the study, again out of an abundance of caution, as our concentrations are not known to be associated with this outcome. All female subjects will receive a urine pregnancy test prior to exposure. Subjects will be informed in the consent document that they will be given the results of the pregnancy test. Although the CO_2 exposure levels used in the study are common in schools, many indoor workplaces, and submarines and spacecraft, subjects will be informed that these levels have been associated with temporary declines in cognitive function. During the 2-hour exposure, subjects will be visually monitored and can speak with the technicians at will. Physician consultation is available if needed. In Phase 2, subjects will be asked to complete a standard online screening form at the Brain Health Institute to confirm eligibility for the fMRI. An MRI screening questionnaire is used to rule out the presence of any medical problems that would cause a risk in the magnet. The participants' answers to the screening questionnaire are reviewed by the scan operator who must have completed Level 2 Safety Training in accordance with CAHBIR Standard Operating Procedures. Claustrophobia in the magnet is limited by excluding subjects with a history of claustrophobia. Subjects are excluded who do not feel they could comfortably lie still in the scanner, or comfortably wear the passive shim mouthpiece. Subjects wear headphones to reduce the sound of the magnet. Minor physical comforts are reduced by use of comfortable pillows for neck and leg support while in the scanner. Communication is maintained with the subject through use of a microphone and headphones. The subject is also given a button to press should they decide to stop the experiment or capture the experimenter's attention. All scans at the Center for Advanced Human Brain Imaging Research (CAHBIR) are screened by the center's MRI technologist. If there is any sign of an abnormality (liberal criteria), the scan data is reviewed by a certified neuroradiologist through a contract with University Radiology Group (URG).
  If the URG neuroradiologist reports that an abnormality may be clinically significant and recommends referral or further evaluation, the study PI will communicate this finding to the participant if it is deemed clinically significant. Note: participants are not shown or told about any potential abnormality until after the neuroradiologist's evaluation to avoid any unnecessary distress given that the majority of observations in a nonclinical young adult population are clinically nonsignificant.

In phase 3 we introduce computer assisted intravital microscopy (CAIM). There are no known risks associated with CAIM, which is very similar to a slit-lamp exam done at routine optometry exams.

- **Certificate of Confidentiality - NA**
- **Provisions to Protect the Privacy Interests of Subjects**
  All data and specimen will be coded by a Study ID. All identifiable information will be stored in REDCap and accessible only to the study team.

F. Potential Direct Benefits to Subjects
There is no direct benefit to study subjects.

5.0 Special Considerations

5.1 Health Insurance Portability and Accountability Act (HIPAA) – NA

5.2 Family Educational Rights and Privacy Act (FERPA) - NA

5.3 Code of Federal Regulations Title 45 Part 46 (Vulnerable Populations) - NA

5.4 General Data Protection Regulation (GDPR) - NA

5.5 NJ Access to Medical Research Act (Surrogate Consent) - NA

6.0 Data Management Plan

6.1 Data Analysis

Means, standard deviations, percentiles and histograms will summarize the distributions of outcome variables. For skewed distributions, transformation such as the logarithmic distribution will be taken to stabilize variance. Initially, multivariate analysis of variance (MANOVA) will be used to examine differences between exposures in the behavioral outcomes measured during exposure and transposed, if necessary, with a random effect to control for correlation between the same outcome across exposure sessions. If a significant effect of exposure is detected, post-hoc analyses will include separate ANOVAs for each outcome again focusing on the effect of the exposure on the outcomes measured at the end of each exposure session. Sensitivity analyses will control for any baseline characteristics in these analyses. Other biomarkers will also be studied using analysis of variance.

6.2 Data Security

All study data will be stored in REDCap is a secure, password-protected web-based application designed to support data capture for research studies. A unique study participant ID will be assigned in REDCap at the time of screening. Specimens, for both Dr. Gow's lab and Baylor University, and SMS data will be coded only with the study ID and Exposure visit number. All data used for analysis will be fully de-identified upon data export from REDCap and stored on an encrypted, password-protected computer network to which only study investigators and staff have access. De-identification of data will consist of removal of all personal identifiers from analytic files. Investigators will keep the data, including links to personal identifiers, until study closure. Collaborators at Baylor University will not have access to identifiers.

In Phase 2, the MRI form is kept in a locked file cabinet and is only accessible by the staff of the Center for Advanced Human Brain Imaging Research and individuals related to Rutgers University who are involved in research safety oversight. CAHBIR has contracted with Flywheel and Google Cloud Services to provide an encrypted cloud-based data transfer, preprocessing, and archiving environment for MRI data, which allows access to numerous automated data quality control and processing steps, with 24/7 support. All neuroimaging data will be archived on Google Cloud via the contract with Flywheel.

In Phase 3, the data collected using CAIM will be stored in university secure restricted Box folder and will only be accessible to authorized research personnel.

6.3 Data and Safety Monitoring - NA

6.4 Reporting Results

A. Individual Subjects' Results

Individual results will not be given to the subject because no clinically relevant results will be obtained during the study. In Phase 2, T1-weighted scan pictures of the brain are shared with the participants who complete the MRI.

All scans at the Center for Advanced Human Brain Imaging Research (CAHBIR) are screened by the center's MRI technologist. If there is any sign of an abnormality (liberal criteria), the scan data is reviewed by a certified neuroradiologist through a contract with University Radiology Group (URG). If the URG neuroradiologist reports that an abnormality may be clinically significant and recommends referral or further evaluation, the study PI will communicate this finding to the participant if it is deemed clinically significant. Note: participants are not shown or told about any potential abnormality until after the neuroradiologist's evaluation to avoid any unnecessary distress given that the majority of observations in a nonclinical young adult population are clinically nonsignificant. Incidental findings are shared with the subject only if the neuroradiologist determines that they warrant further evaluation or treatment.

B. Aggregate Results

Research results will be posted on the EOHSI website.

C. Professional Reporting

Results will be presented at scientific conferences and published in peer-reviewed literature.

D. Clinical Trials Registration, Results Reporting and Consent Posting

The study is a Basic Experimental Study Involving Humans (BESH) and will be registered on clinicaltrials.gov. As required, the consent form and results will be posted.

6.5 Secondary Use of the Data - NA

7.0 Research Repositories – Specimens and/or Data

Remaining specimens at Rutgers University will be stored for future research only by the study investigators; they will not be distributed to others. The specimens will be stored in -80°C freezers at EOHSI Room 232. Freezer temperatures are monitored remotely, and notifications are sent in cases of malfunction. Access to the freezer room is controlled by key card.

8.0 Approvals/Authorizations - NA

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