University of Washington Center for Clean Air Research

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CENTER ABSTRACT
EPA Grant Number:
Center Title: University of Washington Center for Clean Air Research (UW CCAR)
Investigators: Sverre Vedal (Center PI; svedal@uw.edu), Matt Campen, Tom Jobson, Joel Kaufman, Tim Larson, Joe Mauderly, Jake McDonald, Michael Rosenfeld, Paul Sampson, Lianne Sheppard, Christopher Simpson, Adam Szpiro, Timothy VanReken, Michael Yost
Institutions: University of Washington, Seattle, WA; Lovelace Respiratory Research Institute and University of New Mexico, Albuquerque, NM; Washington State University, Pullman, WA
EPA Project Officer: Stacey Katz/Gail Robarge
Project Period: December 1, 2010 – November 30, 2015
Project Amount: $8,000,000
RFA: Clean Air Research Centers
Research Category: Air Quality
Description:
Objectives: The UW CCAR is focused on the cardiovascular health effects of near-roadway pollution, a complex mixture of particle, vapor and gas phase components that vary by vehicle emission source, road surface, extent of physical aging and the type and degree of atmospheric processing and photochemical reactions. This exposure scenario is not only known to be of considerable health importance, it also serves as a prototypical case for developing research approaches to dealing with multi-pollutant exposure-effect relationships. Our aim is to integrate exposure, epidemiological, toxicological, clinical, and statistical sciences to study cardiovascular hazards of fresh and aged roadway emissions and significantly advance our understanding of the components and reaction products that cause these effects.
Approach: Investigators from four institutions are joining in a multi-disciplinary effort to study health effects of near-roadway pollution in line with current efforts to move from a single-pollutant to a multi-pollutant perspective. The Center consists of five highly-integrated research projects and two facility cores (including a Biostatistics Core) that together have the following six tasks: (1) to characterize real-world near-roadway pollutant concentrations, particle size distributions and chemical composition; (2) to simulate realistic contrasting near-roadway multi-pollutant exposure atmospheres for laboratory animal and human studies; (3) to identify cardiovascular and immunologic effects and the pathogenic mechanisms of near-roadway exposures using animal models; (4) to identify cardiovascular and immunologic effects of near-roadway exposures in human clinical studies; (5) to identify effects of long-term exposure to traffic-derived particles and gases on sub-clinical measures of cardiovascular disease and DNA methylation in a multi-ethnic population; and (6) to develop a statistical and methodological framework for studying health effects of multi-pollutant mixtures.
Expected Results: The Center program of research addresses at least three of the research questions posed in the RFA: (1) pollutant health effects in a multi-pollutant context; (2) biological mechanisms underlying health effects; and (3) exposure-response relationships. Identifying the most hazardous components of near-roadway exposures will allow more focused, coordinated and effective air pollution health policy based on sound science to reduce health impacts of this multi-pollutant exposure.
Supplemental Keywords: atherosclerosis, epidemiology, toxicology, transportation

ABSTRACT: Project 1
EPA Grant Number:
Project Title: Exposure mapping – characterization of gases and particles for exposure assessment in health effects and laboratory studies
Investigators: Michael Yost (PI; airion@uw.edu), Timothy Larson, Christopher Simpson, Thomas Jobson, Timothy VanReken
Institutions: University of Washington, Seattle, WA; Washington State University, Pullman, WA
Project Period: December 1, 2010 - November 30, 2015
Project Amount: $1,129,676
EPA Project Officer: Stacey Katz/Gail Robarge
RFA: Clean Air Research Centers; **Research Category:** Air Quality
Description:
Objectives: Roadway-source air pollutants encompass a diversity of chemicals, including both particulate and gas phase components which are transformed by chemical and physical reactions as they age in the environment. Consequently, human exposures to air pollutants can range from relatively un-aged to highly aged components that vary with respect to particle size and the chemical composition of particle and gas phase components. This proposal is will employ mobile and fixed site monitoring to assess both gas and particle components of these pollutants as they age from roadway sources to population areas, for a more comprehensive understanding of the seasonal and spatial variability in the concentration and composition of air pollutant exposures within MESA-Air cities. The main project objectives are: (1) Characterize spatial and temporal gradients of selected air pollutants along roadways and within neighborhoods in MESA cities using a mobile platform; (2) Measure spatial variation in concentrations of selected air pollutants at two-week average fixed sites in coordination with the mobile measurements. (3) Characterize aging of air pollutant components transported from roadway sources to neighborhood receptor locations; and (4) Provide detailed characterization of laboratory exposure conditions available for toxicology testing, and identify likely conditions that mimic those found in urban settings.
Approach: We will (1) use mobile monitoring with an instrument platform designed to measure concentrations of particles and gases while continuously on the move. These data will be used by the Biostatistics Core to develop multivariate spatial models of selected roadway-source air pollutants for use in health studies, and to characterize the aging of air pollutant components as they are transported from sources to populated areas; (2) use passive monitoring at approximately 20 stationary sites in each of the four MESA cities to measure concentrations of coarse particles, gases (O_3, SO_2, NO, NO_2), and selected volatile organic compounds (VOCs). These measurements will be used in conjunction with the mobile measurements to develop multivariate spatial models of selected roadway-source air pollutants; (3) characterize the laboratory exposure conditions available for toxicology testing, and identify likely conditions that mimic those found in urban settings. This will be achieved by deploying the same instruments used in the mobile monitoring platform, along with LRRI instruments and additional high sensitivity mass-spectrometer instruments only available for the laboratory facilities (Aerosol TOF-MS for particles and PTR-MS for VOCs).
Expected Results: In this project, we will (1) develop multivariate spatial models of selected roadway-source air pollutants for use in health studies; (2) characterize the aging of roadway source air pollutant components as they are transported from sources to populated areas; (3) characterize the laboratory multi-pollutant atmospheres for toxicology testing, to help describe physical and chemical transformation processes occurring in the laboratory and to help determine the comparability of conditions generated by in the laboratory to those observed in the field.
Supplemental Keywords: exposure science, community exposures, chemical transport

ABSTRACT: Project 2
EPA Grant Number:
Project Title: Simulated Roadway Exposure Atmospheres for Laboratory Animal and Human Studies
Investigators: Jacob D. McDonald (PI; jmcDonal@lrri.org), Joe L. Mauderly, Melanie Doyle-Eisele, Tim Larson
Institution: Lovelace Respiratory Research Institute, Albuquerque, NM
EPA Project Officer: Stacey Katz/Gail Robarge
Project Period: December 1, 2010-November 30, 2015
Project Amount: $1,214,000
RFA: Clean Air Research Centers
Research Category: Air Quality
Description:

Objectives: This Project will develop inhalation exposure atmospheres for animal and human laboratory studies, with the primary objective of simulating environments containing key components of roadway emissions and the products of environmental factors that transform them. The exposures will help determine air contaminants that cause or potentiate the toxicity of roadway emissions or confound interpretations based on roadway proximity alone. Our hypotheses are that combined gasoline and diesel motor vehicle emissions toxicity decreases when transformed in the atmosphere. We further hypothesize that background air and nonexhaust roadway emissions (road surface dust, tire and brake wear material, inorganic ions, metals, and ozone) do not contribute significantly to roadway-associated cardiovascular morbidity, nor do they potentiate the morbidity associated with roadway emissions. The animal and human toxicology projects will utilize the experimental exposure atmospheres generated in this project to determine the relative potency of different simulated roadway environments, and thus test hypotheses regarding causal components and combinations. The results of the animal studies will be used to select atmospheres for confirmatory human inhalation studies.
Approach: We will develop novel inhalation exposure atmospheres that simulate near roadway and downwind motor vehicle emissions after physical and chemical transformation in the air. Physical aging will be used to convert ultrafine particles that are emitted from the tailpipe at 10-20 nm to agglomerated particles that are 100-150 nm. A third atmosphere will utilize an irradiation chamber to chemically transform motor vehicle emissions. Non-tailpipe roadway emissions will be simulated by a road dust atmosphere with and without motor vehicle emissions. Urban background will be created to include a mixture on non-motor vehicle exhaust that includes ozone, hydrocarbons, metals and inorganic ions (sulfate/nitrate). Urban background potency will be compared against and in combination with motor vehicle emissions. We will define the biological potency of each atmosphere based on lipid peroxidation in ApoE$^{-/-}$mice (further described in Project 3).
Expected Results: We will elucidate the important characteristics that define toxicity resulting from roadway emissions and their interaction with background air. We expect that fresh whole exhaust containing ultrafine particles and vapor will confer the most potent atmosphere. These results will be confirmed in both rodent and human studies.

Supplemental Keywords: particulate matter, volatile organic compounds, carbon monoxide, ozone, chemical transformation, motor vehicle, road dust

ABSTRACT: Project 3
EPA Grant Number:
Project Title: Cardiovascular Consequences of Immune Modification by Traffic-Related Emissions, Project 3
Investigators: Matthew Campen (Co-PI; mcampen@salud.unm.edu), Michael Rosenfeld (Co-PI; ssmjm@uw.edu), Amie Lund, Jacob McDonald
Institution(s): University of New Mexico and Lovelace Respiratory Research Institute, Albuquerque, NM and University of Washington, Seattle, WA
EPA Project Officer: Stacey Katz/Gail Robarge
Project Period: December 1, 2010 – November 30, 2015
Project Cost: $834,357
RFA: Clean Air Research Centers; **Research Category**: Air Quality
Description:
Objectives/Hypothesis: Traffic-related emissions are associated with the incidence and progression of acute and chronic cardiovascular sequelae in human population studies. Such phenomena of near-roadway health effects have yet to be characterized toxicologically. Because of overlapping issues related to noise, socioeconomic status, ethnicity, etc, there is a need to better understand the biological plausibility that fresh mixtures of vehicular emissions have a more potent than expected impact on human health. We hypothesize that the complex mixtures produced by traffic are inherently more toxic due to the combined presence of both particulates and volatile organic emissions. Furthermore, we hypothesize that emissions-induced oxidation of certain endogenous phospholipids, presumably from the pulmonary surfactant, can stimulate the activity of immune cells through such receptors and in turn promote the invasion of existing vascular lesions.

Approach: This project will use complex roadway mixtures as generated and characterized in the laboratory. In **Aim 1**, we will ascertain 1) the potentiating effects of physical and photochemical aging on fresh emissions and 2) interactions of vehicular emissions with pertinent copollutants (ozone, road dust), both in terms of driving systemic vascular oxidative stress**. In Aim 2**, we will examine effects of the emissions-induced oxidative modifications to endogenous phospholipids, in terms of activating immune-modulating receptors such as LOX-1, CD-36, TLR-2, and TLR-4. This Aim will utilize transgenic models to examine the roles of these receptors, as well as characterize the lipidomic alterations in various tissues. Lastly, **in Aim 3**, we will further explore the role of specific immune cell populations as participants in the innate and adaptive responses to emissions-induced phospholipid modifications. In this Aim, we will utilize mouse models of immunodeficiency, including SCID and B-Cell deficient models. Additionally, we will pursue bone-marrow transplants from mice lacking those receptors described in Aim 2 to mechanistically establish the involvement of the oxidatively-modified phospholipids.

Expected Results: Findings will 1) indicate the most potent combinations of urban roadway and background copollutants in terms of vascular toxicity and 2) detail the role of the immune system in mechanistically driving the systemic effects of inhaled pollutants.

Supplemental Keywords: (do not duplicate terms used in text): coronary artery disease, oxidized phospholipids, atherosclerosis, particulate matter, volatile organic compounds, carbon monoxide, ozone

ABSTRACT: Project 4
EPA Grant Number:
Project Title: Vascular Response to Traffic-Derived Inhalation in Humans
Investigators: Joel D. Kaufman (PI; joelk@uw.edu), Tim V. Larson, Jacob MacDonald, Michael Rosenfeld
Institution: University of Washington, Seattle, WA; Lovelace Respiratory Research Institute, Albuquerque, NM
EPA Project Officer: Stacey Katz/Gail Robarge
Project Period: December 1, 2010 – November 30, 2015
Project Amount: $940,545
RFA: Clean Air Research Centers; **Research Category**: Air Quality
Description:
Objectives: Air pollution exposures are associated with ischemic heart diseases. Recent observations demonstrate that traffic-related air pollutants acutely trigger increased arterial reactivity, vasoconstriction, and increased blood pressure in humans and animals; these effects can be used to understand both acute and chronic health effects of air pollutants. This project will use controlled clinical exposures to test the hypothesis that traffic-derived (e.g., diesel and gasoline engine) aerosols exert vascular effects in human subjects, and provide insight into the most toxic components and underlying mechanisms.
Approach: We will use a well-characterized human exposure facility, customized to reflect findings in Center Projects 1-3, to examine effects of simulated roadway-derived exhaust in a double-blind, randomized, controlled crossover experiment. Building on data derived from animal studies and exposure characterization studies (Projects 1-3) in Center years 1 and 2, we propose clinical experiments nested within a crossover trial to be largely conducted in Center years 3 and 4. In healthy subjects, we will test whether a traffic-derived laboratory-generated high-potency pollution atmosphere, as suggested through other Center projects, causes an increased vascular response (brachial artery vasoconstriction and increased blood pressure) compared with both a roadway-derived exposure of hypothesized lower potency and with filtered air.
We also propose several nested aims to examine hypotheses in healthy volunteers in order to better understand the epidemiological observations of both acute (triggering) and chronic (pro-atherogenic) air pollution effects. These nested aims include: whether specific exhaust-related monocytic gene expression effects are mediated by lipid peroxidation and blocked by an anti-oxidant; whether traffic-related pollutants' vasoconstrictive effects are increased in subjects with a common SNP variant in the gene coding for lipoxygenase-15; and whether lymphocyte DNA hypermethylation in specific genes is increased with exposure to simulated roadway-derived exposures.
Expected Results: By coordinating closely with Center Projects 1-3, we will determine whether specific aspects of traffic-derived exposure (primary vs. secondary organics, particulate vs. gases, spark-ignition vs. diesel engine vs. a mixture) enhance the human vascular response to pollutants. We also will learn about biological mechanisms involved in human health effects from traffic pollutants. These studies will have important implications for air pollution regulatory efforts and suggest new approaches for the prevention of cardiovascular health effects.

Supplemental Keywords: cardiovascular health, particulate matter, motor vehicle

ABSTRACT: Project 5
EPA Grant Number:
Project Title: Effects of long-term exposure to traffic-derived particles and gases on subclinical measures of cardiovascular disease in a multi-ethnic cohort
Investigators: Sverre Vedal (PI; svedal@uw.edu), Joel Kaufman, Timothy Larson, Michael Yost, Adam Szpiro, Paul Sampson, Lianne Sheppard
Institution: University of Washington, Seattle, WA
EPA Project Officer: Stacey Katz/Gail Robarge
Project Period: December 1, 2010 – November 30, 2015
Project Amount: $1,357,012
RFA: Clean Air Research Centers; **Research Category**: Air Quality
Description:
Objectives: Exposure to air pollution, especially particulate matter (PM), is consistently linked to cardiovascular disease (CVD) in epidemiological studies. Larger effects of long-term PM exposure are seen with improved exposure estimates. Traffic is a major source of air pollution and an important contributor to CVD; integrating refined traffic exposures into an epidemiologic study of air pollution and CVD would be an important advance. The primary objective of this project is to estimate the effect of individual-level exposure to traffic-derived air pollution on measures of CVD in the Multi-Ethnic Study of Atherosclerosis and Air Pollution (MESA Air) using novel exposure estimation methods and incorporating on-road, in-transit exposure estimates.
Approach: This project has three tasks. First, a multi-pollutant exposure prediction model for roadway-associated air pollution will be built that incorporates complex spatial information on primary and secondary traffic-derived particles and gases. This model will yield: 1) city-wide exposure surfaces for traffic-derived air pollution components for four study cities, and 2) distributions of traffic-derived air pollutant estimates for various roadway types and traffic conditions in each city. Second, individual-level exposure estimates will be developed for traffic-derived air pollutants, utilizing the models built under the first objective. We will enhance and validate these estimates using a personal, residential, and in-vehicle monitoring campaign, including real-time data logged GPS tracking, in a subset of 144 MESA Air participants. Third, the effect of individual-level exposure to traffic-related air pollution, including on-roadway exposures, on longitudinal vascular outcomes (including left ventricular mass and retinal arteriolar diameter) and DNA methylation will be estimated in a cohort of over 4,000 MESA Air participants.
Expected results: This project will transform MESA Air from its current focus on $PM_{2.5}$ into a multi-pollutant study that can meaningfully investigate the impact of traffic-derived air pollution on cardiovascular health using a source-to-exposure approach. We will integrate data on traffic-derived pollutants from the novel, state-of-the-art mobile monitoring campaign (Project 1) into a multi-pollutant exposure model that incorporates participant-specific time-location information. The relationship between traffic exposure and change in measures of CVD will be assessed in a large and well-characterized cohort, making this project the first application of a multi-pollutant approach to a large-scale air pollution epidemiology study. Results will, in turn, assist policymakers in taking a multi-pollutant approach to controlling adverse health impacts of air pollution exposure.
Supplemental Keywords: epidemiology, volatile organic compounds, atherosclerosis

Description of the University of Washington Center for Clean Air Research (UW CCAR)

Introduction:

The UW CCAR is focused on the cardiovascular health effects of near-roadway pollution. This exposure scenario is not only known to be of considerable health importance, it also serves as a prototypical case for developing research approaches to dealing with multi-pollutant exposure-effect relationships. Near-roadway pollution is a complex mixture of particle, vapor and gas phase components that vary by vehicle emission source, road surface, extent of physical aging and the type and degree of atmospheric processing and photochemical reactions. Improvements in our understanding of near-roadway exposure effects from taking a more realistic and sophisticated multi-pollutant perspective would be expected to translate into more effective air pollution policies to reduce the public health burden of these exposures.

Our immediate aim in this Center is to integrate exposure, epidemiological, toxicological, clinical, and statistical sciences (a five-pronged approach) to both examine the cardiovascular effects of fresh and aged roadway emissions and, simultaneously, to advance our understanding of the components and reaction products that cause the effects. The general notion underlying our approach is that exposure, especially long-term exposure, to specific but as yet poorly characterized traffic-derived primary (fresh) and/or secondary (aged) organic particles and gases (and mixtures of these) cause or hasten development of cardiovascular disease. We maintain that this can be rigorously tested and putative causal components can be identified using an interlinked, multidisciplinary investigational strategy and applying statistical methods aimed specifically at the multi-pollutant problem.

Our approach builds on insights gained from our work to date that: 1) fresh, whole vehicular emissions can drive the oxidation of lipids and inflammation in atherosclerotic regions, an effect that is dependent on both particulate and gaseous components of the emissions; 2) the combination of fresh gasoline engine emissions, which are relatively low in PM mass and high in volatile organic compounds, and the higher PM-containing diesel engine emissions lead to synergistic increases in vascular lipid peroxidation; 3) acute exposure to diesel exhaust increases vascular tone; and 4) long-term traffic exposure has important effects on cardiovascular health as reflected in cardiac structure and function, specifically left ventricular mass, and on microvascular disease, as reflected by retinal artery narrowing. These experimental and observational findings enable us to focus our biological hypotheses and choice of endpoints to take the next significant step forward in understanding this multi-pollutant conundrum and providing information useful in directing actions to mitigate the health burden associated with proximity to busy roadways. While obtaining a complete understanding of the near-roadway exposure-cardiovascular disease relationship and identification of all causal pollutant species and their combinations is overly ambitious at this point, we have high confidence that the work we propose is an absolutely necessary and timely step toward that end.

Our Center is designed to address at least three of the six of research questions posed in the RFA [(i) pollutant health effects in a multi-pollutant context; (ii) biological mechanisms underlying health effects; (iii) exposure-response relationships] and has

relevance for another two [(iv) susceptible subpopulations; and (v) origin, transportation and transformation of multi-pollutant constituents]. The goals, objectives and approaches to be used in this Center are described below.

Goal:

The ultimate goal of the UW CCAR is to understand the physical and chemical features of exposures to near-roadway mixtures, including their interactions and concentration-response relationship, that cause or exacerbate cardiovascular health effects.

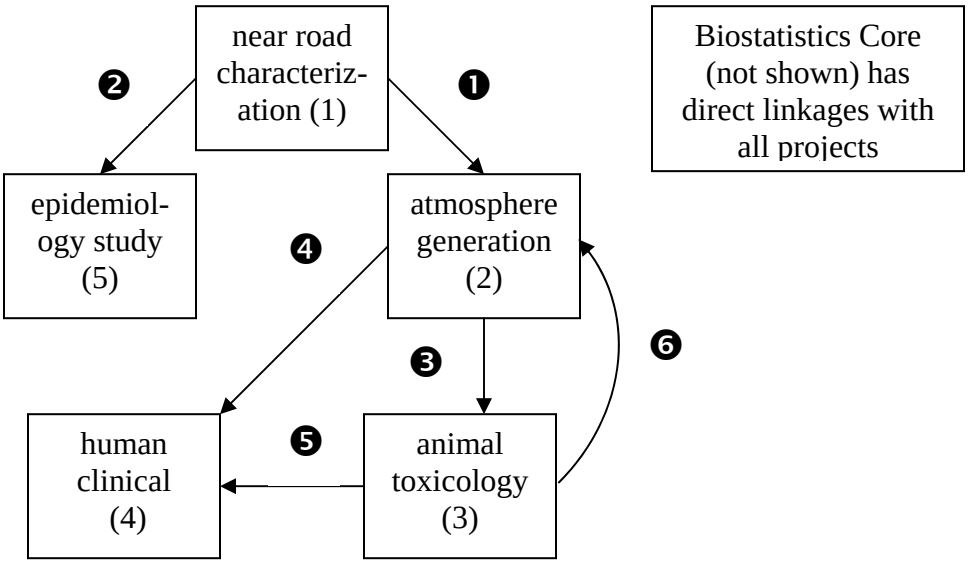
Objectives and Approach:

General:

The University of Washington team, with its expertise in exposure assessment and estimation in clinical exposure studies, toxicology and air pollution epidemiology including major field studies, and in biostatistical methods development and application, is joining with the Lovelace Respiratory Research Institute (LRRI) and University of New Mexico team, with its well-developed and unique expertise in development and characterization of laboratory-generated exposure atmospheres and in toxicology, and with the Washington State University (WSU) team with its unique pollutant monitoring capabilities, to tackle the challenging problems entailed in taking a multi-pollutant approach to investigating air pollution health effects. The proposed research applies a highly-integrated, multi-disciplinary approach to the common theme of near-roadway exposures and cardiovascular health effects. While a multidisciplinary approach to air pollution health effects research is not original, our application of the highly interlinked five-pronged approach we propose is unique and is likely to make substantial progress in the use of a multi-pollutant approach to improving understanding of air pollution health effects.

The Center we propose is clearly an interdisciplinary enterprise, although the discipline-oriented divisions (projects and cores) are somewhat arbitrarily defined because of the strongly interwoven nature of all of the projects. The use of a human clinical study in the experimental setting to extend and validate our exposure atmosphere-related toxicological findings is particularly noteworthy, but that is only one example. Health endpoints center on common themes across the animal and human experiments and the laboratory and population studies. The field characterization component informs the evolution of experimental exposure atmospheres. The Biostatistics Core develops methods for dealing with multivariate data of a nature common to all three health studies (projects 3, 4 and 5), methods that are intended to find ready application by the other centers created under this RFA and by other investigators attempting to unravel effects of mixed exposures.

The primary interactions (black circles with numbers) between projects (project #) are depicted in the figure below and detailed in the descriptions of approaches that follow with corresponding numbers. This is followed by itemization of the overall objective of each component of the UW CCAR and the approach taken to reaching that objective.



Objective 1 (project 1). Characterize real-world near-roadway pollutant concentrations, particle size distributions and chemical composition.

Approach. We first to use a combination of mobile and fixed monitoring in cities of our cardiovascular cohort (MESA Air) to obtain detailed characterization of roadway-related gaseous pollutants and particles as they are transported from roadway to residential neighborhoods. The mobile monitoring will also obtain information regarding on-road pollutant characteristics that will be used in conjunction with in-transit (on-road) personal monitoring (in Project 5, part of interaction ❷). Also, laboratory-generated atmospheres that are systematically manipulated (in Project 2, interaction ❶) will be analyzed in detail to assess correspondence with real-world scenarios to allow the most realistic exposures to be generated for our toxicologic (Project 3, interaction ❸) and human clinical (Project 4, interaction ❹) studies. Monitoring data will be used by the Biostatistics Core to build exposure models of ambient concentrations of roadway pollutants at the individual residence level for use in our MESA Air cardiovascular cohort study (Project 5).

Objective 2 (project 2). Simulate realistic contrasting near-roadway multi-pollutant exposure atmospheres for laboratory animal and human studies.

Approach. We will generate, under controlled laboratory conditions, the different exposure atmospheres that result from roadway emissions (tailpipe and non-tailpipe [i.e., road dust]) and their transformation in order to allow a systematic comparison of effects in our toxicologic (Project 3, interaction ❸) and human clinical (Project 4, interaction ❹) studies. The mixed motor vehicle exposures will mimic near-roadway exhaust and emissions after atmospheric aging and simulating exhaust after it undergoes nucleation and agglomeration. Atmospheres will also be chemically transformed in an irradiation chamber and altered by background air or ozone by integrating co-exposures with these constituents. Background air mixtures will be simulated with combined inorganic ions,

metals, and secondary organics that exist in proportions that are measured near roadways (in Project 1, interaction ❶).

Objective 3 (project 3). Identify cardiovascular effects and pathogenic mechanisms of near-roadway exposures in animal models.

Approach. This project will utilize the contrasting exposure atmospheres generated (Project 2, interaction ❸) to investigate the modifying effects on fresh roadway emission-induced vascular oxidative stress of roadway mixture transformations, both chemical and physical, and pertinent co-pollutants (e.g., ozone, road dust). Several mouse models will assess the universality of the vascular response; transgenic mouse models and models of immunodeficiency will also be employed. A robust primary endpoint of aortic lipid peroxidation, and several secondary endpoints, will be used. A first round of toxicologic studies will generate information on relative toxicity of the atmospheres compared (interaction ❻) that will be used in the subsequent more mechanistically-oriented toxicology studies and in the human clinical studies (Project 4, interaction ❹).

Objective 4 (project 4). Investigate vascular response to combustion-derived gases and particles in humans.

Approach. We will use our established inhalational exposure facility and pertinent experience in human exposure studies to conduct double-blind, controlled crossover studies, randomized to order, to test hypotheses that contrasting near-roadway exposure atmospheres have different vascular effects in human subjects. These studies will be largely launched in Center Year 3, guided by findings generated in Center Projects 1-3, with exposure atmosphere generation led by the Project 2 team (interaction ❹). Primary endpoints include acute exposure responses on brachial artery vasoconstriction, increased circulating endothelin, and systolic blood pressure; others endpoints in sub-studies include measures of lipid peroxidation and related gene transcription and epigenetic changes. Susceptibility will be assessed using an established genetic polymorphism in the lipoxygenase ALOX15 and biological mechanisms confirmed with a pharmacologic blocker of lipid peroxidation, alpha-lipoic acid.

Objective 5 (project 5). Identify effects of long-term exposure to traffic-derived particles and gases on sub-clinical measures of cardiovascular disease in a multi-ethnic cohort.

Approach. Using the data from the mobile and fixed site monitoring campaigns in the MESA Air cities (Project 1, part of interaction ❷), a land-use regression-based universal co-kriging multivariate prediction model (Biostatistics Core) will be generated to estimate concentrations of contrasting components of the near-roadway mix at individual residences of MESA Air cohort subjects. Indoor concentrations will be estimated from an infiltration model. Mobile on-road measurements will allow estimation of on-road exposures. Together, these data will be coupled with participant-specific time-location information to yield individual-level predictions of exposure to near-roadway pollutants. Personal monitoring and a location-tracking campaign in a subset of MESA Air participants will be used to validate estimated on-road exposures. Progressively more sophisticated roadway mixture exposure metrics, ranging from estimated ambient residential concentrations to estimates that incorporate on-road exposures, will be used to estimate effects of long-term exposure in the MESA Air cities on subclinical cardiovascular disease, using both the atherosclerosis endpoints in the main MESA Air

study, as well as the novel and important outcomes of left ventricular mass and retinal artery microvascular dimensions. Finally, epigenetic effects of near-roadway exposures on DNA methylation will be investigated in more exploratory analyses.

Objective 6 (included in the Biostatistics Core). Develop a statistical and methodological framework for comparing the associations between contrasting air pollutant mixtures and health endpoints in health studies.

Approach. This research consists of five parts: (1) develop a statistical model to predict the multi-pollutant spatial field of concentrations for components considered as part of the mix; (2) identify a small number of linear combinations of pollutant concentrations that can be used to represent the range of pollutant mixtures; (3) develop methods for inference in a health effects model based on an *n*-dimensional predicted exposure that includes corrections for measurement error; (4) implement a framework for interpreting effect estimates from the health effects model; (5) apply this methodology to the MESA cohort analysis (Project 5).

The Biostatistics Core. The overall objective of this Core is to support the database management and statistical needs of all Center activities.

Approach. This Core will provide the statistical support that enables Center investigators to incorporate rigorous statistical analyses and advanced statistical methods into their research. It will engage in interdisciplinary interactions, support all projects, ensure careful attention to proper application of statistical methods, and foster scientifically relevant methodological development. This will be accomplished through the following activities: (1) advise Center projects on data management and compilation; (2) ensure quality statistical design and analysis of Center research; (3) implement novel statistical methods that are required for Center projects (objective 6); (4) identify additional statistical methodological research that will advance Center projects; (5) communicate and disseminate Center findings.

Results and Benefits

Outputs from this center project include: (1) a prototypical application of the multi-pollutant approach to health effects research; (2) a novel characterization of near roadway air pollution mixtures for exposure atmosphere generation and exposure estimation; (3) an integrated set of experimental and observational findings using both animal models and humans; and (4) a novel method to assess relative health impacts of different mixtures of a near roadway multi-pollutant exposure. Outcomes include: (1) advancing the methodology for multi-pollutant health research; and (2) allowing more focused, coordinated and effective air pollution policy based on sound science to reduce public health impacts of air pollution exposure.

Project 1: Exposure Mapping – Characterization of Gases and Particles for Exposure Assessment in Health Effects and Laboratory Studies

OBJECTIVES

Air pollutants encompass a diversity of chemicals, including both particulate and gas phase components. Diesel and gasoline vehicle exhaust, ozone and nitrogen oxides are among the highest priority air toxics nationally and a number of studies have reported significant associations between traffic density or roadway proximity and various health outcomes[1-4]. Vehicle sources emit complex chemical mixtures including both particulate and vapor phase components, which are transformed by chemical and physical reactions as they age in the environment. Consequently, human exposures to air pollutants can range from relatively un-aged to highly aged components that vary with respect to particle size and the chemical composition of particle and gas phase components. This proposal is unique in that it will employ mobile and stationary site monitoring to assess the multi-pollutant mixture of both gas and particle components as they age from roadway sources to population areas, for a more comprehensive understanding of the seasonal and spatial variability in the concentration and composition of air pollutant exposures within MESA-Air cities.

The main project objectives are:
1. Characterize spatial and temporal gradients of selected air pollutants along roadways and within neighborhoods in MESA cities using a mobile platform;
2. Measure spatial variation in concentrations of selected air pollutants at two-week average stationary sites in coordination with the mobile measurements.
3. Characterize aging of air pollutant components as they are transported from roadway sources to neighborhood receptor locations;
4. Provide detailed characterization of laboratory exposure conditions available for toxicology testing, and identify likely conditions that mimic those found in urban settings.

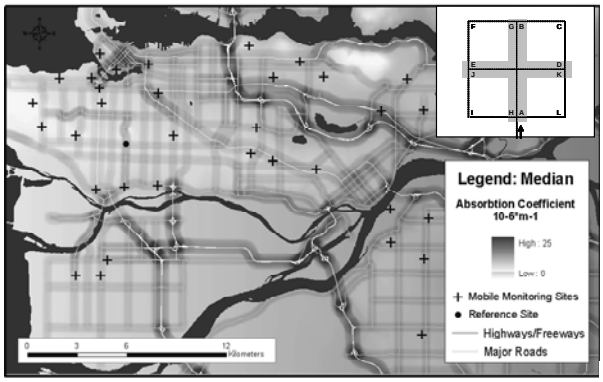
BACKGROUND AND APPROACH

Objective 1: Characterize spatial and temporal gradients of selected air pollutants along roadways and within neighborhoods in MESA cities using a mobile platform.

We will use mobile monitoring with an instrument platform designed to measure concentrations of particles and gases while continuously on the move. The platform will visit a prescribed route in each of four cities. In addition, we will deploy a stationary "central" site in each city to capture variations over time during the mobile monitoring periods. Measuring the short term co-variability in a suite of pollutant concentrations over relevant spatial gradients using a mobile platform provides information on sources that are difficult to disentangle with longer-term measurements of fewer pollutant metrics at a few stationary sites[5-9]. Mobile monitoring is also less resource intensive than deploying many stationary site continuous monitors at many different locations. Our goal is to use these mobile derived measurements to inform the spatial and spatio-temporal exposure prediction models as described by Project 5 and the Biostat Core.

Prior studies

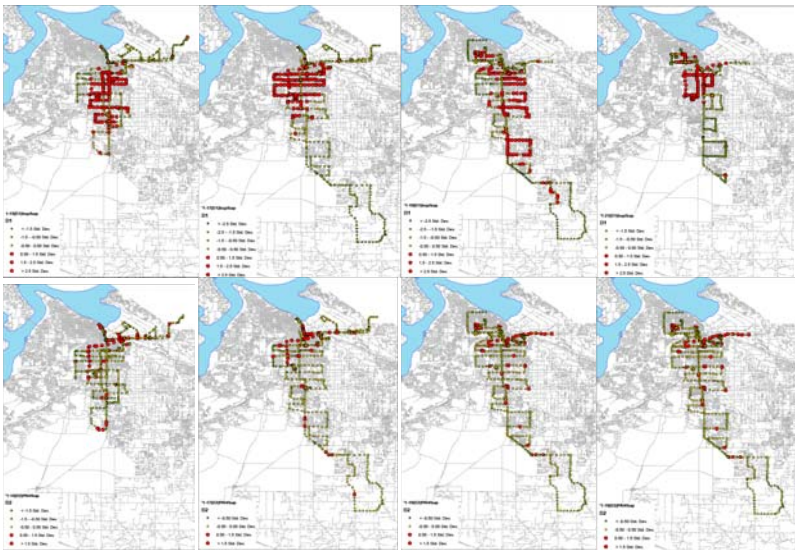
In collaboration with the University of British Columbia we recently used a mobile monitoring platform to collect a set of spatially resolved black carbon measurements during the summer in Vancouver, British Columbia[10]. These measurements were incorporated into a geographic information system (GIS)-based model framework in order to identify the location of elevated,



persistent summer afternoon levels of black carbon that are consistent with the presence of traffic emissions and that are not captured by a relatively dense regulatory ambient monitoring network. The success of this approach as applied to traffic-related pollutants required defining the frequency distribution of short-term measurements sampled within a relatively small area defined by various traffic route intersections. The sampling vehicle followed a cloverleaf pattern (A through L in figure above) and the set of 10 second averages collected along the cloverleaf comprised the frequency distribution assigned to that location. The median value measured within a given cloverleaf was not affected by occasional plumes from polluting vehicles, but was correlated with nearby total traffic levels. Its LUR-derived surface (robust $R^2 = 0.56$) is shown in the figure above. Other surfaces including the mean and 90th percentile were also computed and were more strongly associated with truck traffic than general traffic.

Work currently in progress by our group in Tacoma, WA has expanded this approach to include simultaneous measurements of light absorption coefficient (σ_{ap}), light scattering coefficient (σ_{sp}) and particle-bound PAHs during the afternoon rush hour in the winter. This is the most challenging season to study traffic impacts in Tacoma, given that the southern part of Tacoma is also affected by wood smoke in the winter and that afternoon commuting hours overlaps to some extent with early light-up of residential woodstoves.

	σ_{sp}	σ_{ap}	PAHs
F (observed value)	1.491	3.088	4.450
F (critical value)	0.388	0.402	0.414
p-value (one-tailed)	0.170	0.003	<0.0001



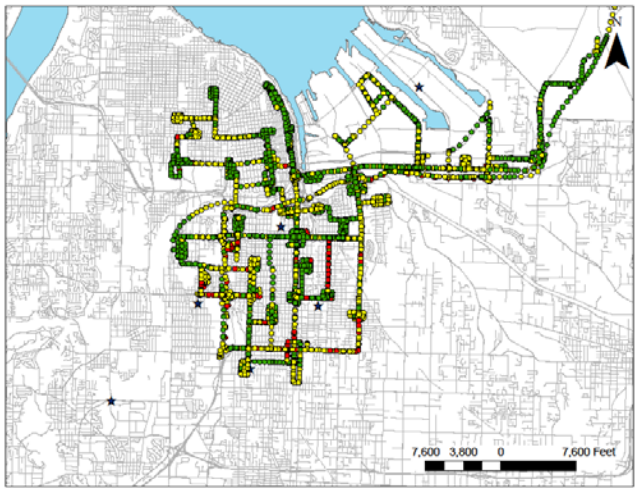
The adjacent table (Table 1) summarizes measurements taken at 16-18 cloverleaves across Tacoma. To reduce day to day variability, all measurements were adjusted to nearby 30-minute average stationary site values of either σ_{sp} or σ_{ap}, the latter values also used to adjust the PAH measurements. We then compared the between-day variance of the adjusted medians at a given cloverleaf with the between-site variance of the adjusted medians across all sites using Levene's test. These results indicate that median σ_{ap} and particle-bound PAH values vary by more than a factor of three on average across sites relative to within sites across different sampling days. The p-values using Bartlett's test are similar. However, the median σ_{sp} values do not vary as much across Tacoma compared with variation over days at a given site. We hypothesize that these difference are due to regional woodsmoke varying over a large spatial

scale without persistent and strong spatial gradients due to trafficked roads. To test this hypothesis, we analyzed all measurements taken during both afternoon and evening sampling routes in Tacoma. These measurements included not only the cloverleaves, but the larger scale routes designed to capture residential wood burning impacts.

The above figure shows the location of the highest 20% of factor scores on four different days (columns) for two separate multivariate features (rows) extracted from the set of these three variables using principal components with varimax rotation. The top row shows a feature with factor loadings for $\sigma_{sp}>\sigma_{ap}$ > PAHs and factor scores highest at night in the residential areas of south Tacoma but also present in lower amounts during afternoon periods. This principal component feature overlays the areas of highest woodstove use. The bottom row shows a feature with factor loadings for PAH > $\sigma_{ap}>\sigma_{sp}$ and factor scores highest during the afternoon near heavily traveled roads and the Port of Tacoma. Similar patterns were found using features and contributions derived from a two-source Unmix model.

Proposed Sampling Routes

Mobile monitoring will be conducted in 4 MESA-Air cities: Baltimore, Winston-Salem, Chicago, and Los Angeles. Monitoring will take place at approximately 40 locations selected to represent different strata (tertiles) of ambient NOx pollutant levels based on previous MESA- Air sampling and spatial-temporal modeling of pollutants. A sampling period will comprise measurements taken both in the afternoon and again in the evening at approximately 15 cloverleaves. Therefore, to sample at 40 locations we need three sample periods. Each cloverleaf will be visited a minimum of three sampling periods per season over two seasons (heating, non-heating). Therefore we expect to need approximately 9 sample periods per season or 18 total sampling periods in each city.



The adjacent figure shows the location of the total set of σ_{sp} afternoon measurements in Tacoma. In addition to visiting the intersection cloverleaves, we naturally obtain measurements while traveling between these locations. We therefore will also select the monitoring locations in each MESA city with these additional between-cloverleaf routes in mind. Specifically, we have travel demand model shapefiles for all the MESA cities and will use this information to identify well traveled commuter routes. The sampling routes and cloverleaf locations will be established prior to sampling and visited following one of two different specific sequences. The particular sequence for a given sampling period will be chosen at random.

Pollutant Measurements

The table below (Table 2) summarizes the instruments to be used on both the mobile platform as well as at the accompanying stationary-site used for temporal adjustments.. As in our previous studies, the instrumentation will be placed inside a vehicle and connected to a sampling manifold inlet placed out an otherwise sealed window. We have chosen this set of measurements for

several reasons. First, in our experience the detection limits, response times and power requirements are adequate. The gases are routinely measured by the AQS network and therefore provide useful contrasts in their own right. Particle light scattering (mildly heated inlet) also is highly correlated with $PM_{2.5}$, another pollutant routinely monitored by EPA in these cities. The particle size distribution (also from the heated inlet) is known to vary among primary pollution sources and also known to change rapidly due to physical aging (see discussion in Objective 3).

Table 2: Measurements during mobile platform campaigns

	Metric	Instrument
Mobile platform	Light scattering coefficient	Radiance Research Nephelometer M903
	Light absorption coefficient	Magee Aethalometer Model AE51
	Particle-bound PAHs	EcoChem PAS 2000
	Particle optical diameter (0.25-30 μm)	Grimm laser aerosol spectrometer 1.109
	Particle optical diameter (0.3-10 μm)	AeroTrak 9303
	Particle number concentration and mean particle mobility diameter (0.025 – 0.3 μm)	Grimm NanoCheck 1.320
	O3, NO, NO2	2B Technologies Models 202, 400 and 401
	CO	Langan T15v
	Location	Garmin GPS
Stationary Site	Light scattering coefficient	Radiance Research Nephelometer M903
	Light absorption coefficient	Magee Aethalometer Model AE51
	Particle optical diameter (0.3-10 μm)	AeroTrak 9303
	Particle number conc. (>0.025 nm)	P-TRAK UPC w/ diffusion screen
	CO	Langan T15v

The stationary site will be used for temporal adjustments as described in the next section. Its location will depend upon the spatio-temporal model predictions of NOx. Ideally it will be co-located with an EPA site. It must be located such that it captures the diurnal impacts of traffic without being too near the road to be overly influenced by individual tailpipe plumes. The EPA siting guidance for neighborhood scale sites is therefore appropriate.

Initial Data Processing

The mobile platform measurements will first be adjusted for temporal variation using the fixed site data. In prior studies discussed earlier we have used a simple ratio adjustment as follows: $C_{15\,\mathrm{sec}}^{adjusted} = C_{15\,\mathrm{sec}}^{mobile}\left\{\frac{C_{\mathrm{overall}}^{fixed}}{C_{30\,\mathrm{min}}^{fixed}}\right\}$. The subscripts refer to the signal averaging time. The 30-minute stationary site average was used in prior work and was computed at the center of the time window of a moving average updated each minute. The overall average used in the adjustment is that for all measurements taken at the stationary site during the season's sampling campaign.

Based upon the results described earlier, the stationary site values for σ_{ap} are adequate for adjustment of the PAH values, given that we do not plan to measure PAHs at the stationary site. For adjustment of the size distribution measurements, we initially plan to use CO values as a conservative tracer and marker of dilution. These values also will be used in assessment of particle aging as discussed in Objective 3. We will explore other stationary site averaging times and adjustment strategies as part of this research by comparing the between-day variance of the adjusted medians at a given cloverleaf (or commuter route) with the between-site variance of the adjusted medians across all cloverleaf sites as described earlier. After adjusting for prevailing

wind patterns for sites adjacent to major roads (Wilton et al, submitted), if the F values are too low, then that particular variable will be flagged.

Frequency distributions of the adjusted mean and median values, as well as other quantiles, will be generated for each cloverleaf and for appropriate length sections of the commuting links between the cloverleaves. These will then be provided to the Statistical Core for use in spatial models of outdoor levels as described in Project 5.

Objective 2: Measure spatial variation in concentrations of selected air pollutants at two-week average stationary sites in coordination with the mobile measurements.

We will use passive monitoring to evaluate concentrations of coarse particles, inorganic gases (O_3, SO_2, NO, NO_2 and selected volatile organic compounds (VOCs) at approximately 20 stationary sites in each city. Passive samplers were originally developed for personal exposure assessments, but due to their low cost and ease of use, they have been increasingly used to monitor a broad range of inorganic gases and VOCs in ambient air[11,12]. Passive samplers have been tested as an alternate method to conventional air sampling methods, such as auto GC and canisters. Specifically, the passive samples have been tested for their stability and effectiveness in the laboratory and community-based outdoor settings to measure low concentration ambient VOCs. Air pollutant concentrations measured with the passive samplers will be used by the statistics core, in conjunction with the mobile measurements described above, to develop multivariate spatial models of selected roadway-source air pollutants for all four cities, use in the health studies described in project 5. These models also will be of use in health studies undertaken by the MESA-Air grant in all four cities and for coarse PM currently being developed in Winston-Salem and Chicago by an EPA Coarse-PM grant. In addition, we shall explore correlations between selected inorganic gases and VOCs measured with passive samplers at the stationary sites, with features of the multi-pollutant mixture identified via the mobile monitoring (objectives 1 & 3) including relative contributions of gasoline vs diesel emissions and fresh vs aged roadway-source emissions. The analysis of coarse mode particles is particularly relevant, since recent toxicological testing indicates that coarse particles both near and far from roadways can create inflammatory responses which are separate from and cardiac-ischemic injury responses caused by ultrafine mode (<100nm) particles[13].

To select these stationary locations where passive monitors will be placed, we shall use the city and season-specific spatial models for NOx, developed by the MESA-Air project to identify regions of low, medium and high outdoor NOx concentrations in each city. We choose NOx as an indicator of roadway-source air pollution because it exhibits stronger gradients near roadways and therefore shows greater within-city spatial variation than fine particle mass[14,15]. Sampling sites will be selected in each of these three tertiles of NOx concentrations, and may include private residences (e.g. homes of MESA study participants) and public buildings (schools, libraries, government buildings etc). Passive samplers will also be located at the fixed site described in objective 1 (this site is used to provide temporal adjustments for the mobile monitoring). Ideally this site will be collocated with an EPA site. A secondary aim of the passive monitoring is to characterize changes in the composition of selected roadway-source air pollutants as they are transport from sources to more distant home receptor locations in support of Objective 3. These composition changes can be captured by ensuring that the stationary sites are selected to include some locations nearby and just downwind of major roadways, and other sites that are more distant, both upwind and downwind of the roadways. Sampling devices will

be simultaneously deployed in each of four cities (Los Angeles, Chicago, Baltimore and Winston-Salem), for one 2 week period in each of 2 seasons over a year 2 and 3 of the study.

Rationale for VOC selection: VOCs are a major component of the roadway-source multi-pollutant mixture, frequently accounting for a far greater fraction of the total mass of roadway-source emissions than the condensed phase of the emissions (i.e. particle mass). The roadway-source VOC mixture contains a variety of compounds know to be toxic and/or carcinogenic to humans including benzene 1,3-butadiene, aldehydes and various solvents. Several toxicological studies have indicated that the vapor phase component of vehicle emissions plays a significant part in the adverse health effects of vehicle emissions[16]. The health effects cause by the vapor phase component can be distinctly different from, and sometimes more important than, the health effects attributable to the particle phase components.
The various sources that contribute to the roadway-source multi-pollutant mixture each emit unique combinations of VOCs. Furthermore, chemical transformation of the VOC mixture(s) occurs as the fresh roadway emissions age and undergo (photo)chemical transformation during transport down-wind from roadways to receptor locations, giving rise to new multivariate VOC features that provide information on the degree of aging/transformation of the original roadway emissions. Recently, factor analytic approaches have been successfully applied to ambient VOC measurements to resolve contribution from these different sources[17,18]. Major source contributions to ambient VOCs include gasoline and diesel exhaust emissions, evaporative emissions from fuel, biogenic emissions and aged vehicle emissions. Additionally, in specific locations major industrial point sources can dominate VOC concentrations[19].

The primary specific aim of the passive monitoring in this project (Project 1) is to measure spatial variation in concentrations of selected roadway-source air pollutants. These data will be used in conjunction with the mobile measurements to develop multivariate spatial models of selected roadway-source air pollutants for use in health studies. A secondary aim of the passive monitoring is to characterize changes in the composition of selected roadway-source air pollutants as they are transport from sources to more distant home receptor locations. We have selected a suite of VOCs *a priori* with these two specific aims in mind. The selected compounds are shown in table 3, along with typical ambient concentrations of these compounds, measured at the EPA PAMS network monitoring site in Rutgers, NJ. We reviewed existing source apportionment studies[20,18] and selected compounds that were found to be useful for quantifying contributions from primary roadway source emissions (including separating gasoline and diesel exhaust e.g. Benzene, toluene, xylene, iso-pentane, nonane, decane, undecane), compounds useful for distinguishing roadway and non-roadway derived VOCs (e.g. isoprene, n-pentane), and compounds enriched in aged roadway emissions. Some authors have also used ratios of specific BTEX compounds to determine the extent of photochemical processing of VOC mixtures[21,22]. Additional criteria for selection of compounds included (i) sufficiently high anticipated ambient concentrations that the compound would be reliably detected, and (ii) acceptable collection efficiently on the activated-charcoal based OVM sampler over the two week period. Thus ethane, propane and polar VOCs including aldehydes were not included in our list of target VOCs because they are not efficiently collected using the OVM sampler.

As described in objective 4, chemical characterization of the controlled atmospheres at LRRI and UW will occur in year 1 of the study. The LRRI atmospheres will include both fresh and aged (photochemically transformed) vehicle emissions. Therefore, we will have the opportunity to refine our list of target VOCs based on the results from objective 4. We shall also consider the

option to co-locate passive samplers containing alternative sorbents (e.g. XAD, PUF, DNPH-treated silica) at a selection of the stationary sites to enable collection of some of the more polar VOCs that are likely to be useful markers for the contribution of aged roadway-source emissions.

Table 3. List of roadway source VOCs to be measured and typical ambient concentrations for these compounds.

Compound	Mean Ambient Conc. (ppbv), Rutgers, NJ[1]	Rationale for selection
Benzene	0.15	Major driver for VOC cancer hazard; present in vehicle exhaust and biomass smoke
Isoprene	0.59	Biogenic emissions and aged aerosol
Toluene	0.72	Gasoline (GE) and Diesel exhaust (DE)
n-Decane	0.04	Enriched in DE vs. GE
Nonane	0.03	Enriched in DE vs. GE
2-methylpentane	0.17	*Enriched in GE vs. DE*
m-Xylene	0.16	Enriched in DE vs. GE
Undecane	0.03	Enriched in DE vs. GE
i-Pentane	0.68	Enriched in biogenic and evaporative emissions
n-Pentane	0.37	Enriched in biogenic and evaporative emissions
o-Xylene	0.06	Enriched in DE vs. GE

A passive sampler described by Ott, Cyrs, and Peters[23] will be used to monitor ambient coarse particles PM10-2.5 at the stationary sample site locations. This method uses a modified version of the passive sampler developed by Wagner and Leith [24] employing a 12-mm-diameter cover glass as the collection substrate, and applying low-cost light microscopy combined with digital image analysis to determine particle surface loading by size. Particle mass concentrations are estimated from surface loadings and calculated deposition velocities, with an assumed particle density ρ_p and aerodynamic shape factor S_D, but with the volumetric shape factor S_V derived directly from microscopy. With this technique, the most sensitive parameter (S_V) in calculating mass concentration from microscopic data is based on the individual characteristics of measured particles. Analysis will use at least 500 particles counted in at least 10 sample fields to assure robust statistics. In addition, the samplers will be housed in small shelters to protect the samplers from precipitation and reduce the effects of wind on the particle deposition characteristics[25]. Laboratory and field tests have been conducted to evaluate the precision and accuracy of this method[23,26]. The estimated limit of detection (LOD) for this method when applied for 2-week sampling periods is 0.9 ug/m^3 with a analytical CV of ~12%. We also will co-locate passive samplers at the EPA reference sites where active air sampling is used to collect PM10 and PM2.5 samples, to evaluate potential bias and accuracy of the passive samplers. Field evaluation of the passive sampling method indicates that PM10-2.5 exposures can be assessed at a scale of a few kilometers or less[26].

Ogawa passive samplers will be used to collect NO_2, NO_x, SO_2, and O_3 (one two-sided sampler for NO_x/NO_2/ SO_2 and a second single sided sampler for O_3). The samplers will then be analyzed at the University of Washington by ion chromatography to measure NO_2, SO_2, and O_3, and by ultraviolet spectroscopy (UV Spec) to measure NO_x Ambient concentrations of each pollutant will be calculated using the equations provided by Ogawa & Co. The Ogawa sampler has an LOD of 0.3 ppb, 0.5 ppb, and 0.4 ppb for NO_2/ NO_x, SO_2, and O_3 respectively, for 2-week

duration samples (Ogawa USA website). We have previously used these samplers successfully in the MESA-Air project[11], employing the same 2-week sampling protocol that will be used in the current study.

The passive VOC samplers chosen for this project are 3M 3500 Organic Vapor Monitor (OVM) badges. OVMs have been found to be reliable passive samplers in other studies[27]. Among the passive samplers, charcoal-based organic vapor monitors (OVMs) primarily have been tested, including recent performance tests with field sampling in Houston, TX and New Orleans, LA[12,27]. For common aliphatic and aromatic hydrocarbons, including benzene and toluene, the OVM samplers were effective for sampling periods up to 2-weeks, and showed no evidence of saturation or analyte breakthrough when used to sample typical ambient concentrations of VOCs. VOC concentrations will be measured by using solvent desorption followed by GC/MS analysis in a modification of the badge manufacturer's recommended procedure. In brief, 2mL benzene-free CS_2 containing appropriate internal standards will be injected onto the sorbent bed using the port in the back of the OVM badge. The badges will be placed on a mechanical agitator for 2 hours to enable extraction of the VOCs from the sorbent bed. The CS_2 extract will then be removed from the passive badge and transferred to a glass autosampler for analysis by Gas chromatography-mass spectrometry (GC/MS). The MS system will be operated in the selected-ion mode to enhance sensitivity for detection of the VOCs. Multiple acquisition windows will be programmed, such that no more than five selected ions are monitored in each window, further enhancing sensitivity for the VOCs. Samples will be extracted and analyzed batch wise (up to 40 samples/batch). Samples will be randomized with respect to the order of sample extraction and analysis, to avoid introducing bias due to variation of instrument response over time into the VOC data. At least 15% of samples analyzed will be QC samples including filed blanks, laboratory blanks, co-located duplicate field samples and laboratory-spiked samples. We anticipate LODs in the range 0.002-0.01 ppbv. Comparing these anticipated LODs to the typical ambient concentrations listed in table 3, it is clear our procedures will have sufficient sensitivity to detect the target analytes at ambient levels. The LODs were estimated based on the 14-day sampling period for the passive samplers, using the manufacturer's compound-specific effective sampling rates for the OVMs badges (effective sampled volume 0.4-0.8m^3) and an instrumental limit of detection (LODs) for SIM GC/MS of 10ng/mL.

Objective 3: Characterize aging of air pollutant components as they are transported from roadway sources to neighborhood receptor locations.

The combination of mobile monitoring and stationary site monitoring described above will be used to evaluate the characteristics of air pollutants at near-roadway (un-aged) and neighborhood receptor (partially aged) locations. In order to account for dilution of the near roadway plume with "background" air, CO will be used as a conservative tracer of urban emissions and results will be normalized to CO concentration. Photochemical age will primarily be marked by the oxidation of NO, and operationally defined as −Log (NO/ NOx).

The comparison of these measurements will provide insight into the degree to which aging modifies the chemical and physical characteristics of the exposures for the participant locations. The metrics collected in mobile monitoring campaigns that will provide indicators of pollutant aging will be: NO/NOx ratios, corresponding local O3 depletion, ultrafine PM/NOx ratios, particle bound PAH/NOx ratios, and ultrafine PM/Fine PM ratios. The mobile monitoring will be conducted on a neighborhood scale (1-10km), and will sample spatially around a stationary

reference site with continuous time-resolved data. The stationary site samples will provide a suite of measurements similar to the mobile measurements.

From the perspective of mobile monitoring, we will structure the mobile monitoring routes to capture these secondary features of pollutant aging. The mobile routes will traverse paths between residential areas and heavily trafficked roads, as defined by the existing traffic demand model nodes used in the MESA-Air modeling of PM and other pollutants. The measurements in-between these node locations will be traversed at least 3 times on different observation days, in an effort to tease-out the aging phenomena. A secondary signal of pollutant aging, observed by the mobile platform will involve ratios of the concentrations of non-reactive CO to the concentrations of more reactive pollutants such as NO, NO2, O3. However, it is somewhat less obvious how to interpret particle size information in this regard. Taking a clue from the CO ratios, we will examine the ratios of different particle size classes relative to CO for various particle metrics. These latter metrics will involve some integration- e.g. the total aerosol volume between 0.1 and 1.0 um (e.g. light scattering coefficient), the particle number concentration or total particle surface area for ultrafine particles (.025-.25um) as deduced from the NanoCheck. In this regard, observing relatively high concentrations of ultrafine particles provides an indicator that the mixture is less aged.

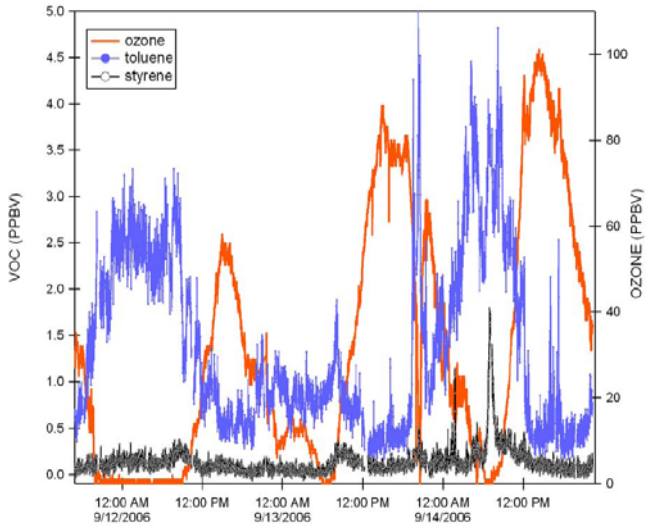
Objective 4. Provide detailed characterization of laboratory exposure conditions available for toxicology testing, and identify likely conditions that mimic those found in urban settings.

Characterization of toxicology lab conditions (Project 2) will be done by deploying the same instruments used in the mobile monitoring platform, along with additional high sensitivity mass-spectrometer instruments only available for the laboratory facilities. We will perform detailed characterization of the exposure atmospheres, using the HR-ToF-AMS and PTR-MS provided by the research team from the Laboratory for Atmospheric Research at Washington State University. These instruments will provide reference measurements, and we will correlate this mass spectrometer data to the suite of measurements collected from the mobile monitoring instruments and to the suite of measurements done at LRRI (see Project 2). We will conduct these measurements at the UW laboratory late in year 2 after it is retrofitted with the aging chamber.

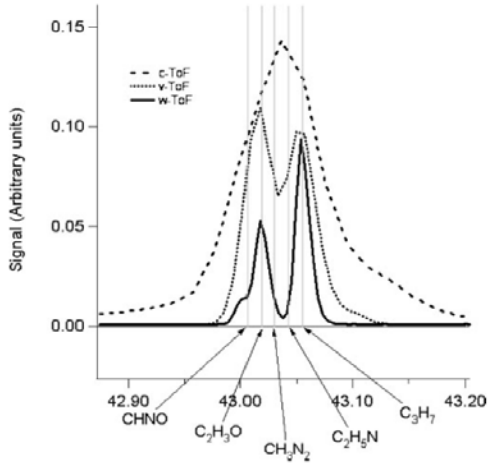
In particular, we will perform a parametric study, where the LRRI and UW laboratories will be asked to systematically vary their exposure system conditions while we analyze the gas and particle compositions of the mixtures with the instruments. We then will conduct a factor analysis of the laboratory data, to decompose the complex signals into different gas and particle component signals and correlate these with the mobile instrument signals. Using these same factor components we also can compare the results of these laboratory measurements to the data obtained in the field studies, in terms of VOC composition, organic and oxygenated aerosol components (OOA and HOA), NO/NO2 ratios, ultrafine PM/NOx ratios, particle bound PAH/NOx ratios, and ultrafine PM/Fine PM ratios. This will help to identify laboratory test conditions that are most similar to the exposures found in near-roadway and residential-receptor locations observed in the MESA-air study cities.

Aerosols will be analyzed with a time-of-flight aerosol mass spectrometer (HR-ToF-AMS) that provides size and chemical composition information. In particular, we will evaluate the HR-ToF-AMS organic aerosols for two components: hydrocarbon-like organic aerosol (HOA), traced by m/z 57 (mainly C4H9+), related to primary anthropogenic urban (and biomass burning) emissions, and oxygenated organic aerosol (OOA), traced by m/z 44 (mainly CO2+), exhibiting a much greater oxygen content mainly due to secondary organic aerosol (SOA) formation through photochemical reactions. The gas phase components in the exposure systems will be analyzed with a Proton Transfer Reaction - Mass Spectrometers (PTR-MS) that yields high sensitivity identification and detection of volatile organic compounds (VOCs), quantifying concentrations into the ppt range.

The PTR-MS has been shown to be an effective and efficient technique for quantifying selected exhaust emission products from on-road vehicles. Mixing ratios deduced by the PTR-MS technique for benzene and toluene are found to correlate very well with GC-FID data from collocated canister samples. Other PTR-MS ion masses represent collections of compounds such as mass 57, 107 and 121. have been found to agree very well with the sum of the MTBE and isomeric butene concentrations derived from GC-FID measurements. PTR-MS mixing ratios showed significant correlations with the comparable C2 and C3-benzene measurements by GC-FID. The adjacent Figure shows an example of time-series of PRT-MS organic measured component data collected by the WSU team in Mexico City.



The High-Resolution Time-of-Flight Aerosol Mass Spectrometer (HR-ToF-AMS) provides the best combination of size resolvability, detection limit, and mass resolving power available for particle research. These capabilities will provide us with significant insight into the nature of organic aerosol materials used in the laboratory studies, and into the conditions that contribute to the evolution of particles as they age. Previous work has shown that AMS organic aerosol mass spectra can be de-convolved to determine oxygenated and hydrocarbon-like organic aerosol (OOA and HOA) components[28] and that in Pittsburgh[29] or Mexico City[30,31], OOA is mostly SOA, and HOA is mostly primary organic aerosols (POA) associated with fresh fossil fuel combustion. The ability to directly correlate measures of particle chemistry with the mass of OOA is a new and unique component of our analysis compared to using total organic aerosol mass (POA + SOA) or OC/EC ratios, which has been done in the few ambient studies looking at this issue in the past[32,33]



While the number of compounds in an aerosol sample and the fragmentation associated with vaporization and electron impact ionization generally prevents species

identification, the mass spectra still provide an enormous amount of information about the origin of the condensed phase material, particularly for the organic compounds that are the focus of much of the work in this center. Compared with other versions of the AMS, the HR-ToF-AMS has a much greater mass resolution that for the first time allows clear resolution between fragments with the same nominal mass/charge (m/z) ratio. For example, several common organic fragments have m/z between 43.0 and 43.1; it is possible with the HR-ToF-AMS to distinguish between pure hydrocarbon fragments (e.g., C3H7+) and those that contain oxygen and/or nitrogen (e.g., C2H3O+ and CH3N2+) (see adjacent Figure). This information will greatly facilitate our efforts to understand the formation of secondary organic aerosol, both in terms of the sources of organic precursor compounds and the mechanisms by which these gases are converted into aerosol material.

Expected Results, Benefits and Outcomes

Characterize spatial and temporal gradients of key air pollutants

This project will characterize spatial and temporal gradient of both gaseous and particulate air pollutants within four of the six MESA-Air cities, targeted to those areas within each city where the MESA-Air cohort resides. The measurements proposed in this Project (Project 1) will complement the extensive stationary site monitoring that has been done as part of the MESA-Air and Coarse PM studies funded by EPA and the NPACT study funded by HEI. The mobile platform and passive, stationary-site measurements described in this project will provide several additional spatial 'snapshots' over an approximately two-week period in each of two seasons in each of the four cities. The mobile platform provides a finer scale of measurement than what has already been done in these cities as well as a richer set of measurement variables. The passive, stationary site monitoring is done on a similar spatial scale to previous work in these cities, but provides an important additional set of species that will, combined with the enhanced particulate matter measurements made on the mobile platform, help to resolve heavy duty versus light duty vehicle impacts.

Provide estimates of long-term air pollutant concentrations for use in health studies

Our previous measurements made over a longer time period as part of the EPA and HEI studies mentioned earlier, in conjunction with EPA stationary site measurements, are currently being used to develop spatio-temporal models of long term exposure to $PM_{2.5}$, NO, NO_2, O_3, OC, EC, black carbon and selected trace elements. Simpler spatial models of $PM_{10-2.5}$ are also being developed. We were careful to include measurements in this project that are directly related to those made in the prior studies mentioned above, including light scattering coefficient (surrogate of $PM_{2.5}$), NO, NO2, O_3, light absorption coefficient (surrogate of black carbon) and optical particle size from 2.5 to 10 μm (surrogate of $PM_{10-2.5}$). This Project will also provide information on other important pollutants, including particle bound PAHs, CO and ultrafine particles and a suite of hydrocarbons, albeit on a shorter time scale. Therefore, this Project will provide additional data that can be directly incorporated into the longer term exposure models described by the Biostatistics Core.

Characterize physical aging and chemical transformation of ambient roadway-source air pollutant mixtures

This Project will provide spatially resolved measurements of several reactive gaseous species, NO, NO2, and O3 as well as a non-reactive tracer of vehicle exhaust, CO. It will also provide spatially resolved measurements of rapidly aging ultrafine particles relative to this same non-reactive tracer. Examining the relevant ratios over space will provide important insights into

near-roadway gradients that would otherwise be simply characterized by an individual species concentration at a few stationary locations. Spatial surfaces provided by the multivariate spatial model described in the Statistics Core will provide new insight into regions of rapid aging and chemical transformation near roadways.

Characterize physical aging and chemical transformation of laboratory generated roadway-source atmospheres to be used in toxicology studies

Results from the enhanced measurement of VOCs and particle composition in the controlled exposure settings (Objective 4) along with the proposed measurements described in Project 2 will help elucidate physical and chemical transformation processes occurring in the controlled laboratory atmospheres. In addition, these measurements will help to determine the comparability of exposure atmospheres generated by LRRI and by the UW. These results will help to test the synergistic hypotheses in Project 3 between high VOC gasoline exhaust and high PM diesel exhaust. If it is indeed the case that the gas/particle interactions drive important health endpoints, then these enhanced measurements are critical to understanding the relevant atmospheric interactions and therefore to rationalizing the animal results in Project 3 with the human endpoints in Project 4.

GENERAL PROJECT INFORMATION

Dr. Michael Yost, University of Washington - Dr. Yost is a professor and the director of the Exposure Sciences program at UW. He received his Ph.D. from the UC Berkeley in Environmental Health Sciences with a minor in Electrical Engineering and Biostatistics. He founded and directs the optical remote sensing (ORS) lab at UW and has published widely on remote sensing measurements of chemical and aerosol contaminants. He currently directs two research projects working with the Puget Sound Clean Air agency and funded by USEPA to evaluate community exposures to ambient air pollutants in Tacoma WA, and to improve source apportionment methods in Seattle WA. Dr. Yost will oversee the Mobile monitoring field program of the project and serve as the PI for Project 1.

Dr. Timothy Larson, University of Washington - Dr. Larson is the Alan and Inger Osberg Professor of Civil and Environmental Engineering at the University of Washington and also holds an adjunct appointment in the Department of Occupational and Environmental Health Sciences. His expertise is in characterization of urban air pollution, exposure assessment of airborne particles and gases, and source/receptor relationships of ambient air pollutants. Professor Larson will assist with development of the mobile monitoring data collection plan and analysis of the mobile monitoring data.

Dr. Christopher Simpson, University of Washington – Dr. Simpson is an Associate Professor in the Department of Environmental and Occupational Health Sciences. He received his Ph.D. from the University of British Columbia in Environmental and Analytical Chemistry, and has a Master's and Bachelor's in Environmental Chemistry. Dr. Simpson's expertise is developing sensitive analytical methods for measuring exposure to airborne contaminants, including chemicals derived from diesel exhaust and wood smoke. Dr. Simpson will oversee the use of passive samplers and the laboratory analysis of the samples.

Dr. Tom Jobson Washington State University – Dr. Jobson is an Associate Professor Department of Civil & Environmental Engineering and a principal member of the Laboratory for Atmospheric Research at Washington State University in Pullman WA. He received his Ph.D. in Chemistry (1994) from York University, Toronto and his B.Sc. in Chemistry from the University

of Victoria, Victoria BC. His research interests and activities include measurement of trace organic gases in the atmosphere, development of trace gas instrumentation, analysis of tropospheric photochemistry, biogeochemical cycling, and assessment of global climate change. Dr Jobson recently performed a study on hydrocarbon profiles over western Houston seeking to better understand the impact of emissions from large petro-chemical operations. Dr Jobson will lead the WSU team in year 1 and 2 that will perform studies to characterize the laboratory exposure systems. They will deploy a high sensitivity Proton Transfer Reaction Mass Spectrometer (PTR-MS) and an Aerodyne High Resolution Time of Flight Aerosol Mass Spectrometer (HR-ToF-AMS). The PTR-MS will perform trace organic gas analysis and the HR-ToF-AMS will perform speciated particulate matter analysis.

Dr. Tim van Reiken, Washington State University – Dr. VanReken is an Assistant Professor in the Department of Civil & Environmental Engineering and a member of the Laboratory for Atmospheric Research at Washington State University in Pullman WA. He received his Ph.D in Chemical Engineering (2004) from the California Institute of Technology, and has a B.S. degree in Chemical Engineering (1997) from the University of Florida. Dr VanReken was a Postdoctoral Fellow from 2004 – 2006 in the advanced study program at the National Center for Atmospheric Research, Boulder, CO. His research interests and activities include sources and formation of biogenic secondary organic aerosols, aerosol/cloud interactions and novel instrument development. Dr. VanReken will assist with the laboratory characterization studies, particularly in the analysis of the HR-ToF-AMS spectra used for chemically speciated particle analysis.

Facilities, Equipment, and Other Resources—Washington State University (WSU)

As a result of a wide range of atmospheric chemistry research projects, WSU has a broad range of sophisticated instrumentation and established facilities to support our field and modeling program. In particular, two instruments, the HR-ToF-AMS and PTR-MS will be used for characterizing the laboratory exposures used for *in vivo* studies. The High-Resolution Time-of-Flight Aerosol Mass Spectrometer (HR-ToF-AMS) from Aerodyne Research, Inc. provides quantitative measurements of size-resolved aerosol composition of non-refractory materials such as organics, sulfates, nitrates, and ammonium. Particle size is determined by measuring the time-of-flight as particles move from a sample chopper into the ionization region. Ions are mass resolved and detected by a high-resolution time-of-flight mass spectrometer.

Proton Transfer Reaction – Mass Spectrometer. The PTR-MS continuously measures selected volatile organic compounds with a response time of less than 1 Hz and with detection limits of ~ 50 pptv. It measures organics in air by ionizing them with H3O+ in a drift tube. The resulting protonated organics are detected with a quadrupole mass spectrometer. The instrument allows very fast measurements of certain organics and is ideal for measuring surface fluxes. The PTR-MS is a highly complementary instrument to the GC-ITMS. The latter is much better suited for chemical speciation (i.e., determining the specific compound being analyzed), but it is rather slow. The PTR-MS is capable of much more rapid measurements, but is generally unable to distinguish between compounds of the same molecular weight. Having both instruments maximizes our ability to characterize the varying gas-phase organic composition.

Facilities, Equipment, and Other Resources— University of Washington DEOHS

ORS Lab: The proposed project will be organized through the Optical Remote Sensing (ORS) laboratory directed by Professor Yost. The ORS lab has a variety of particle generation and

measurement equipment, such as a TSI 3076 Constant Output Atomizer, 3760A/3762 Condensation Particle Counter, diffusion batteries, cascade impactors, and gravimetric filter sampling equipment, a Versatile Air Pollutant Sampler (VAPS), a Stanford Research Systems QMS-300 portable quadrupole mass spectrometer, and a Malvern Mastersizer X particle spectrometer for sizing particles from 70nm to 50um. The ORS lab also has access to a Leica Microsystems optical microscope with a computer controlled stage and automated counting software capable of automated uv-Vis-fluorescence measurements. Two VICI dynacal controlled test atmosphere generators are available for gas generation, along with a Mettler UMX-2 0.1 μg precision Microbalance for calibration and measurement of weights.

In addition to the above analytical instrumentation the lab is equipped with several networked computer workstations with online storage and data sharing capability. All students have access to scientific computing tools such as Matlab and the specialized programs for data analysis such as ArcGIS, GeoDa, STATA, SPSS, SAS, CALPUF View (+CALMET & CALPOST).

EH lab: A key resource for this project will be the facilities of the UW Environmental Health Laboratory (EHL), which was previously directed by Professor Yost and now is directed by Dr. Russell Dills. The EHL has on staff trained Ph.D. analytical chemists for performing advanced analysis of chemical, environmental, and biological samples. Most of the analysis for the passive monitor studies will be performed in the EHL located on the fourth floor of the F-Wing of the University of Washington Health Sciences Center. The EHL has been accredited by the American Industrial Hygiene Association since 1977. It provides analytical support for field research faculty of the Department of Environmental and Occupational Health Sciences, the Washington Department of Labor and Industries, and labor organizations, employers, and workers throughout the state. The TOAC is supported by research grants and income from fee-for-service analyses.

Schedule

Activity	Year 1	Year 2	Year 3	Year 4	Year 5
Characterization of LRRI & UW atmospheres	x	x			
Field sampling, City 1		x			
Field sampling, City 2		x			
Field sampling, City 3			x		
Field sampling, City 4			x		
Development of spatial models				x	x
Factor Analysis of compositional data		x	x	x	

Project 2: Simulated Roadway Exposure Atmospheres for Laboratory Animal and Human Studies

OBJECTIVES

The overarching goal of the UW CCAR is to better define the impacts of roadway-source air pollutants on cardiovascular morbidity. CCAR will advance our understanding of the composition, nature and toxicity of roadway emissions using a suite of integrated approaches. An integral component of the Center is the development of laboratory-generated atmospheres for experimental exposures of animals and humans. This Project will develop these atmospheres, with the primary objective of simulating environments containing key components of roadway emissions and the products of environmental factors that transform them. The exposures will help determine air contaminants that cause or potentiate the toxicity of roadway emissions or confound interpretations based on roadway proximity alone.

Our hypotheses are that combined gasoline and diesel motor vehicle emissions toxicity decreases when transformed in the atmosphere. We further hypothesize that background air and non-exhaust roadway emissions (road surface dust, tire and brake wear material, inorganic ions, metals, and ozone) do not contribute significantly to roadway-associated cardiovascular morbidity, nor do they potentiate the morbidity associated with roadway emissions. This set of hypotheses will be tested in close integration with the exposure characterization, animal toxicology, and human toxicology projects. Specifically, the exposure characterization project will provide enhanced knowledge of air quality in microenvironments having different relationships to roadways, and will permit better specification of the exposure atmospheres to be developed in the laboratory. The animal and human toxicology projects will utilize the experimental exposure atmospheres generated in this project to determine the relative potency of different simulated roadway environments, and thus test hypotheses regarding causal components and combinations. The questions that this project will help to address are:

1. Does agglomeration and physical transformation of particulate motor vehicle emissions alter their toxicity (does size matter)?
2. Does chemical transformation, and formation of secondary organic aerosol from motor vehicle emission precursors, enhance or diminish the toxicity of roadway atmospheres?
3. Do ozone and other background co-pollutants alter or exacerbate the toxicity of motor vehicle emissions?
4. Does road dust, a significant non-tailpipe roadway emission, confer any cardiovascular toxicity that may confound associations with tailpipe emissions?

The hypotheses and supporting questions will be addressed by execution of 3 Specific Aims.

Aim 1: Develop and characterize laboratory-generated exposure atmospheres simulating the key components of near-roadway exposures, including transformed emissions and co-exposures. Importantly, the infrastructure to conduct much of this work is already in place. This existing infrastructure, and its design that permits much of this work to efficiently be conducted in parallel, allows us to conduct a seemingly ambitious program within the resource constraints available. Aim 1 will develop new approaches to conducting inhalation exposures to MVE that represent MVE as it exists at different distances from a roadway. The outcomes of this air are new exposure configurations that mimic near roadway exposure atmospheres to atmospheres that are physically aged or chemically transformed to represent downwind

exposures. The MVE atmospheres will be studied both as near as realistically practical to the point of emission, and after atmospheric aging simulating time-related particle nucleation and agglomeration. Next, the atmospheres will be chemically transformed in an irradiation (smog) chamber. The ability of a representative background pollutant mix to potentiate the effects of roadway emissions will then be determined by detailed analysis and (when possible) comparison to ambient air measurements made in Project 1. The background mix will be simulated by combining inorganic ions, metals, secondary organics volatile hydrocarbons and ozone in realistic proportions.

Aim 2: Conduct inhalation exposures of laboratory animals. Aim 2 will integrate with the animal toxicology project. Building on previous findings that show synergistic increases in mouse vascular response when gasoline and diesel emissions are combined, we will investigate permutations that investigate the near roadway scenarios developed in Aim 1 and define the biological potency based on lipid peroxidation in $ApoE^{-/-}$ mice (further described in Project 3). The results of the animal toxicology studies will also inform this project by providing feedback for iteratively testing the hypotheses and for selecting or modifying exposures for humans and subsequent animal studies focusing on specific biological response mechanisms. The first round of inhalation exposures will generate substantial biological data and important information on the relative ranking and potential interactive effects of roadway emissions. The subsequent exposures of humans will determine comparability across species, and subsequent exposures of animals will refine our understanding of the biological mechanisms involved and point toward specific causal pollutants.

Aim 3: Conduct inhalation exposures of human subjects. Animal toxicology studies will inform the selection of conditions for the experimental human atmospheres. Although the selection cannot be known at this time, we will conduct exposures to humans with the least and most potent atmosphere observed from the animal studies. Thus, we envision evaluating the effects of two atmospheres in the human exposure studies.

BACKGROUND AND APPROACH

On-roadway and near-roadway microenvironments are complex, and include fresh tailpipe and evaporative emissions, non-tailpipe particulate emissions, pollutants in upwind air from distant roadways and many other sources, and the products of atmospheric transformation and transport. Primary tailpipe emissions derive from both diesel- and gasoline-powered vehicles, and to a lesser extent from natural gas-powered vehicles. Older and poorly-functioning vehicles contribute the majority of the emissions. Once in the atmosphere, tailpipe emissions are transformed. These transformations include nucleation and agglomeration of small (nano) particles into larger particles either by self nucleation or combination with other atmospheric constituents. These "physical" transformations may result in a shift from freshly-emitted particles that are < 20 nm in diameter to agglomerated particles of 150 nm or larger, depending on distance from the roadway. Chemical transformations of motor vehicle emissions also occur. This will include reactions of the gaseous and particulate emissions with a range of atmospheric scavengers, including hydroxyl radical, ozone, nitrate radical, etc. These complex reactions will create mixtures different from fresh emissions, and may also form additional particulate matter through reactions that produce secondary organic aerosols. While poorly studied, the highly reactive vapor phase of (especially gasoline) emissions has potential to form substantial amounts of secondary organic aerosols under proper aging conditions. The chemical composition and toxicity of chemically transformed roadway emissions are poorly understood. Finally,

aerosolization of paved road dust by traffic is an important component of roadway emissions that does not originate from tailpipes. In fact, resuspension of respirable-sized road dust may account for the largest portion of the particulate matter "emitted" by most of the well-functioning, contemporary vehicles.

While there have been a number of studies that investigate the impact of motor vehicle (primarily diesel) emissions in the laboratory, these studies fall short of addressing the complexity of the actual exposures that occur close to roadways. The overarching paradigm that Project 2 intends to model for the purpose of better understanding the impact of roadway pollutants on toxicity is shown in Figure 1. The approach considers the key factors that may affect our ability to understand the toxicity associated with exposure to roadway derived pollutants. Figure 1 notably includes a broader view of traffic related emissions than is typically considered. Traffic emissions occur from a variety of vehicle types (diesel, gasoline, etc), and tailpipe emissions are only a part of what comes from roadways. An inclusive approach acknowledges the potentially important contributions from the resuspension of roadway dust that occurs from geological material, settling of ambient pollutants, and tire/brake wear.

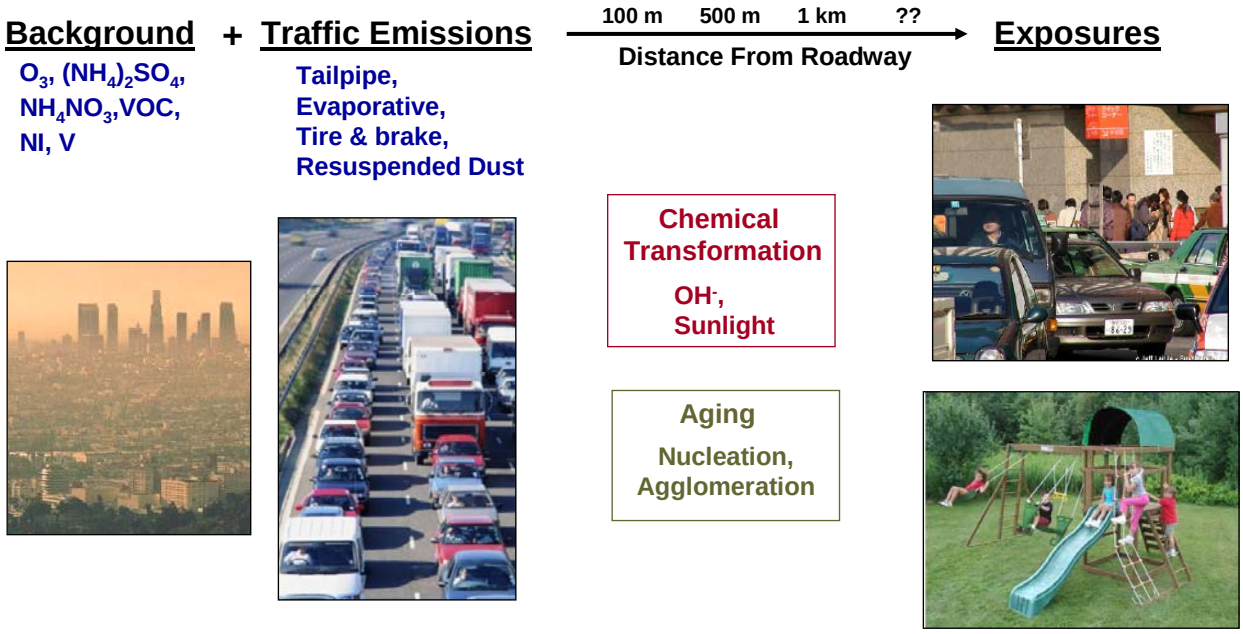


Figure 1. Paradigm for the study of the important factors that may contribute to exposure and morbidity associated with roadway emissions. Traffic emissions, including tailpipe (diesel, gasoline, natural gas) and resuspension of dust, combine with other background co-pollutants. The emissions are physically and chemically transformed as a function of residence time in the air (surrogate for distance from roadway).

In addition to considering the complex and varying emissions that originate from roadways, it is important to understand the potential contribution of interactions between roadway emissions and pollutants in the background air. Neither regional background nor relatively fresh roadway pollutants exist in isolation; most people are exposed to varying combinations of the two depending on time-activity patterns. There is high potential for regional and near-roadway co-

pollutants to interact in either an additive or synergistic manner to affect health. Ozone is a secondary regional pollutant having high potential for interacting with primary emissions. There is some evidence that the product of interactions between motor vehicle emissions and ozone creates products more toxic than either pollutant alone. Roadway-generated pollutants also combine with primary and transformed background pollutants from other sources. In addition to ozone, this background air will include other reactive and oxidized gases, and particulate matter consisting of organic material mixed with inorganic sulfates and nitrates in acidic and neutralized forms. Metals, especially reactive metals, are a small but potentially important component of this background mixture. Crustal materials are also a minor component of regional particulate matter.

In addition to air quality, there are other potentially important factors to consider in relationships between proximity to roadways and health. Some of these include noise and other stressors, genetic variance, and socioeconomic status. Although acknowledged to some extent, these factors have been underserved in most previous studies of air pollution-induced morbidity. The contributions of these factors merit research, but they cannot be addressed within the resources of the proposed project.

The proposed series of atmospheres is shown in Table 1. The experimental groups include MVE (combined diesel and gasoline exhaust at varying ratios), motor vehicle exhaust combined with simulated regional background air, background air alone and MVE after physical and chemical transformations that simulate downwind processing. The experiments will allow us to systematically test our hypotheses concerning the relative effects of roadway and background pollutants on cardiovascular disease, and will provide insight into the importance of interactions between fresh roadway-source and regional pollutants. This approach will provide important information on the potentially synergistic impacts of combinations of major atmospheric components on cardiovascular morbidity. The exposures are related to specific questions below, in the context of the overall Project and Center goals.

1. Does agglomeration and physical transformation of particulate motor vehicle emissions alter their toxicity (ie., does size matter)?

   We will conduct exposures to both fresh MVE (to ensure inclusion of ultrafine and nanoparticles) or after aging of MVE in a residence chamber that simulates agglomeration and nucleation of these particles that occurs downwind from the roadways. Multiple dilutions will be used to enable comparisons based on both mass and number concentration, and to investigate the dose-response of this atmosphere.

2. Does chemical transformation and formation of secondary organic aerosol from MVE precursors, enhance or diminish the toxicity of roadway atmospheres?

   We will conduct exposures to MVE after they are chemically transformed in a smog chamber that simulates reactions occurring adjacent to roadways.

3. Does ozone and other background co-pollutants alter or exacerbate the toxicity of MVE?

   We will conduct exposures to MVE combined with simulated urban background to investigate the exacerbation or potentiation of the cardiovascular effects of MVE alone. If an interaction is observed, we will also conduct exposures to MVE combined with ozone alone, to evaluate its role in the interaction.

4. Does road dust, a significant non-tailpipe roadway emission, confer any cardiovascular toxicity that may confound associations with tailpipe emissions?

   We will also conduct exposures to respirable road dust alone and in combination with MVE.

Table 1. Summary of inhalation exposure atmospheres for Aims 1-2.

	Primary (Fresh)	Aged	Chemically Transformed
MVE	+	-	-
MVE	-	+	-
MVE	-	-	+
Urban Background	-	-	-
Urban Background	+	-	-
Paved Road Dust	-	-	-
Paved Road Dust	+	-	-

This Project is linked to all of the objectives of the Center, but is key to the conduct of the animal (Project 3) and human (Project 4) laboratory exposure studies. The animal studies (Project 3), made possible by this project, will incorporate markers of vascular oxidative damage and atherosclerotic progression. Measurements in the animal studies will parallel and complement the variables evaluated in the human studies. The animal studies will compare the potency of atmospheres representing different exposure scenarios, and the relative potency will serve as a basis for more in-depth mechanistic studies, including assessments of the roles of genetic variance and immune responses on cardiovascular outcomes. The animal studies will also serve to inform the selection of atmospheres for the subsequent experimental human exposures (Project 4), also made possible by this project. The experimental human exposures will include a baseline exposure to mixed motor vehicle emissions compared to the most potent and least potent exposures indicated by the animal experiments. Thus the human exposures will seek to validate the animal results and facilitate translation from the laboratory to population outcomes.

The rationale and specific approach for development of each of these atmospheres are described below. Of special importance is the consideration of exposure concentrations and combinations of the exposure conditions. Project 3 and 4 explain how results of the biological endpoints will be described and compared to evaluate relative potency. The health outcomes will be presumed to reflect the integrated effects of 6 hour daily exposures repeated over the exposure period. Accordingly, the exposure parameters are typically defined as averages integrated over the entire exposure. We will also obtain real-time information on the changes in PM mass, PM composition, particle number, size distribution and gas concentrations. We will thus have the ability to define exposure in multiple ways, and these various exposure metrics will be incorporated in our analysis of relative potency. However, it will still be necessary to define *a priori* target concentrations that are based on specific atmospheric constituents. For example, this decision could be made based on matching concentrations of gas phase components, particle number, particle mass, etc. We propose to begin with particle mass as the primary basis for normalization among exposure-response relationships by initially conducting all exposures at a particle mass concentration of 300 $\mu g/m^3$. Previous results indicate that we should observe

measurable biological responses at that concentration (see Project 3 pilot data), and that we can move downward in concentration from that point to assess dose-response.

Alternative Approaches and Considerations: There are, of course, alternative approaches to assessing the role of traffic and proximity to roadways in air pollution-related morbidity. We considered the possibility of using concentrated ambient particle exposures or previously collected ambient particles. Such approaches could be taken to address the questions we pose by sampling air in various proximity to roadways as previously reported [(1)]. Another approach would be to conduct exposures in the field by taking advantage of traffic tunnels as a real-world source of fresh on-road emissions, bringing a smog chamber and animal exposure unit to the site. Each of these approaches have merit, but our proposed laboratory studies have the great advantage of precise control and knowledge of the exposure conditions. The uncontrollable variables of the field approaches may leave the source/composition/toxicity linkages intractable beyond simple associations. For example, even in traffic tunnels designed to separate vehicles by duty type, we have shown in previous studies [(2)] that the separation is rarely complete (e.g., diesel vehicles in light duty tunnels and vice versa), and the relative contribution of different vehicle types is not reproducible from day to day.

We view laboratory exposures, despite their artificiality, to be necessary for the planned, systematic comparisons needed to test hypotheses about the contrasts in biological effects that may occur at different proximity to roadways. We will investigate the roles of atmospheric processing (both physical agglomeration and chemical transformation) and exposure to co-pollutants that are present in ambient air. We will explore the potential role of respirable road dust, a ubiquitous component of traffic related "emissions" that is seldom considered. These exposures will allow us to extend our previous studies of the cardiovascular effects of engine exhaust and other pollutants in a systematic manner that directly addresses the impacts of proximity to roadways. As noted elsewhere, these studies will also expand the range of the biological responses and mechanisms we have studied, will enable validation by experimental human exposures, and will also provide bridges to our epidemiological studies. Our experimental strategy includes comparisons testing potential for additive, synergistic, and antagonistic interactions between motor vehicle emissions and other air pollutants.

Of course, there are limitations to our approach. We will have precise control over the sources and composition of exposure materials and thus can cleanly test hypotheses; however, we will not be able to evaluate the full range of factors that contribute to near roadway and distant exposures. Within the resources of this project for example, we cannot include natural gas exhaust or exhaust from the latest technology gasoline and diesel engines. We will also not include all potential atmospheric transformation pathways, as the complexity of atmospheric chemistry is well beyond the permutations we could evaluate as part of this work. Our approach is to focus on the final composition of the mixtures, to optimize their representation of exposure atmospheres found in the environment. We will base this on previous experience, published reports on the composition of emissions and ambient air, and the characterization efforts of Project 1.

Another limitation is our approach to comparing exposures, and thus dose, as a basis for judging the relative toxicity of the different exposure scenarios. One logical explanation for findings of increased morbidity near roadways is that the exposures are higher, and that decreased morbidity at distance from roadway is merely a function of reduced exposure due to dilution. This will be tested by conducting dose-response studies with MVE (Project 3). We will then compare effects

of the other atmospheres at an equal particle mass concentration. We can also compare on the basis of particle number. Overall the objective is to investigate relative toxicity. In the environment, traffic emissions decrease due to dilution and/or deposition while they are being atmospherically and chemically transformed. We could elect to model the concentrations that people may be exposed to downwind after the components are transformed. However, there would be significant uncertainty in defining downwind exposures, and that might diminish our ability to directly test the relative potency of the mixtures. It is clear that normalization to any single component of a mixture has inherent limitations in disentangling the chemical components that may be driving toxicity. We will attempt to achieve realistic ratios of the various components, including moderating the gas phase of MVE by denuder technology. To the end, we will at least be able to examine comparisons using multiple indicators, and believe that this work will advance our knowledge significantly.

STUDY DESIGN AND METHODS

Aim 1: Develop and characterize laboratory-generated exposure atmospheres simulating the key components of roadway exposures, including transformed emissions and co-exposures. This work will utilize existing infrastructure to develop new methods and information on the transformation of roadway combustion emissions. Data analysis and statistical support for comparing the composition of the atmospheres are described in the Biostatistics Core. The exposure atmospheres will be normalized to common particle concentration of 300 $\mu g/m^3$, with the exception of dose-response studies for fresh motor vehicle emissions that will be conducted at 10, 30, 100, and 300 $\mu g/m^3$. Gas phase concentrations are also important, and are described below.

Motor Vehicle Emissions (Diesel + Gasoline Engine Emissions)

Background: The proportional contribution of gasoline emissions to the health burden of vehicle emissions is uncertain, yet there is growing evidence that gasoline emissions should not be ignored. Of course, there are myriad potential ratios of diesel and gasoline emissions in the environment, especially considering the huge variability in emission rates and compositions among the on-road fleet. Before using our diesel source for experiments, we confirmed that an operating mode could be selected that yielded emissions falling well within the range of those generated from on-road engines, both in vehicles and on test stands. Our gasoline engine emissions are generated from an engine obtained from a 1996 General Motors truck. The engine has approximately 100,000 miles, and has been studied extensively alone [(3)] and in combination with diesel emissions.

Our pilot data reported in Project 3 shows that combined MVE synergistically increase lipid peroxidation (TBARS) in $ApoE^{-/-}$ mice exposed to other exposure atmospheres, including gasoline and diesel exhaust alone at the same respective concentration. Those data were generated at a gasoline:diesel PM ratio of 1:6. This scenario represents an environment that is more heavily impacted by diesel traffic than gasoline traffic, even when considering the likely difference in PM emissions rates from diesel vehicles compared with gasoline. For the studies proposed here, we intend to conduct studies that match average diesel:gasoline PM ratios observed in Los Angeles during source apportionment studies near roadways [(4)] reported diesel powered vehicles contributing approximately three-quarters of the motor vehicle derived PM near roadways in Los Angeles. For our proposed studies, the PM will thus be composed of a range of diesel and gasoline proportions (4:1, 9:1, 14:1 diesel:gasoline particulate matter ratios)

that will be screened in initial short term (7 day) exposures for their potency. The most potent combination will be selected for subsequent follow-on studies.

Approach

Diesel Engine Exhaust: Diesel exhaust will be produced from a single-cylinder, 5500-watt, Yanmar diesel-engine generator using Number 2 Diesel Certification Fuel (Chevron Phillips Chemical Co., Borger, TX) and 40-weight motor oil (Rotella T, Shell, Huston, TX) as described by [5]. Electrical current is pulled from the engine to provide a constant load (90%) during operation. Extensive preliminary characterization has been conducted on this exhaust generator to allow the emissions to be placed in the context of emissions found in previous laboratory and environmental samples [5].

Gasoline Engine Exhaust: Exhaust will be generated from a 1996 General Motors 4.3-L V6 gasoline engine (mileage ~100 x 10^3) equipped with stock exhaust systems (including muffler and catalyst) obtained from in-use vehicles (Chevrolet S-10 light-duty pickup trucks) purchased in Albuquerque, NM. Non-oxygenated fuel is obtained from a local distributor, and each batch of fuel is characterized upon receipt to define cetane number, sulfur content and aromatic content. The engine is operated on an to eddy current dynamometers on the California Unified Driving Cycle as previously described [3].

Reduction of Exhaust Gases: Our previous studies have investigated cardiovascular toxicity of whole engine exhaust, exhaust mixtures, or exhaust with the particulate removed. One limitation of these previous studies is the artificially high concentrations of gaseous co-pollutants that accompany exhaust from the tailpipe. While it is recognized that these atmospheres also contain artificially high amounts of particulate matter, the relative proportion of gaseous compounds is larger than what is typical in ambient air. To address this, and we will use a denuder technology that will reduce all of the gaseous components while permitting the particulate fraction to pass through. We have found this technology, reported previously by [6], to reduce all of the gaseous components of MVE vehicle exhaust by 90 % or greater with only minor particle loss. For a 300 ug/m^3 particle concentration for example, it is anticipated that the gaseous concentrations will be ~0.5-1 ppm for NOx, 1-5 ppm for CO, and 0.2 ppm for hydrocarbons. These concentrations are a much better reflection of the ratio of gases to particulate matter in ambient air then what we have previously used for engine emissions studies (5; 3).

Atmospheric Aging of Motor Vehicle Exhaust: The ability of dilution conditions and aging to alter the particle size through nucleation and agglomeration has been demonstrated both in the laboratory and as a function of proximity to a roadway [7,8]. Depending on the technology and operating condition, fresh MVE will include large numbers of nanoparticle condensates that are less than 30 nm in diameter. In ambient air and in the laboratory, these particles will undergo nucleation and agglomeration within minutes to transition the particles to the accumulation mode (~100-150 nm). There is much speculation that the smaller particle size may have increased toxicity, although this has not been tested directly with motor vehicle emissions. These studies will evaluate the potential for agglomeration of motor vehicle nanoparticles to decrease the toxicity.

Approach

Fresh emissions will be delivered to the exposure chambers with immediate and rapid dilution to quench agglomeration. The exposure chamber will be placed as close as practical to the combined MVE, with exhaust and exposure atmosphere temperature being the primary determinant of allowable proximity. In order to create an atmosphere of atmospherically transformed motor vehicle exhaust, the MVE will be transported to a 6 m^3 chamber having a 20

minute residence time. The particulate material will agglomerate during this residence time to a predicted particle size of 150 nm (from an initial particle size of 15-20 nm; 3). The residence time and dilution conditions will be iteratively optimized to meet this particle size.

Chemical Transformation of Motor Vehicle Exhaust

Background: In recent years there has been more and more evidence that chemical transformation of hydrocarbons in the atmosphere leads not only to ozone and gas phase oxidants, but also to the formation of secondary organic aerosol. The formation of secondary organic aerosol from gaseous precursors has been well documented for model compounds such as the terpenes [9] and simple aromatics [9]. There have been few studies of the chemical transformation of motor vehicle emissions, and even fewer of the role of chemical transformation on the toxicity of these emissions. Importantly, the reactive nature of especially gasoline engine exhaust hydrocarbons leads to potentially significant chemical transformation in the atmosphere. These reactions would include primarily hydroxyl radical initated oxidations, but may also include reactions with ozone and other oxidants. The resulting transformation would include not only a modification of the organic content of the particulate phase, but also formation of new secondary organic aerosol from the oxidation of the gaseous hydrocarbons. We have hypothesized that the chemical transformation of fresh emissions may decrease the potency of the material. This hypothesis is founded primarily on the fact that many studies have shown decreased toxicity downwind of roadways. It would be counterintuitive to suggest that downwind chemical transformation would increase toxicity substantially. However, this is an important question to address considering the substantial change in composition of motor vehicle emissions downwind from their source.

Approach

Secondary organic aerosol can be formed in laboratory irradiation (smog) chambers when the reactants have sufficient reactivity. We have recently reported on the conduct of inhalation toxicology studies of secondary organic aerosol from biogenic [9,10,11] and aromatic [12] reactants. Those studies used a "continuous flow stir reactor" irradiation chamber that provides a continuous production of secondary organic aerosol that can be used for inhalation exposures. The chamber includes a bank of 40 lights that span the UV-A and UV-B range. Humidity and temperature are maintained inside of the Teflon lined reaction chamber. The reactants in this chamber take ~6 hours to come to an equilibrium reaction condition. Equilibrium is achieved while reactants continue to enter (and exit) the chamber. The equilibrium in the continuous flow stir reaction chamber ensures that the formation of reactants is stabilized and produces a consistent aged atmosphere over periods of time that permit reproducible inhalation studies to be conducted.

For the proposed studies, we will characterize the behavior of mixed motor vehicle exhaust in the exposure chamber to assess the formation of secondary organic aerosol and gaseous oxidants. Based on the evaluation of chemistry and secondary aerosol production, the final reaction and dilution conditions will be defined to provide a total particle concentration of 300 $\mu g/m^3$. The exposures will include other pollutants in some cases. As we have done previously [10,11,13], we will remove the excess ozone selectively using a spent honeycomb carbon denuder.

Urban Background Atmospheres

Background: In addition to carbon, the primary components of ambient particulate matter are sulfate and nitrate, termed secondary inorganics because they are mostly reaction products

resulting from the oxidation of SO_2 (derived from fossil fuel combustion) and NOx (primarily derived from mobile sources). This oxidation produces sulfuric and nitric acids that are subsequently neutralized, primarily by ammonia. Sulfate may exist in PM as sulfuric acid simultaneously with neutralized sulfuric acid, but nitrate only exists in the neutralized form because nitric acid is a gas and ammonium nitrate is a particle. There are substantial seasonal and geographic differences in the contribution of both sulfate and nitrate to ambient PM. When expressed as a fraction of PM mass, sulfate contributes substantially more to total respirable PM in the eastern and industrial midwestern United States compared with the western (especially California) and upper midwestern United States. In contrast, the western United States (especially California), has higher PM contributions from nitrate. While metals comprise a small portion of PM mass, they represent a potentially biologically-important component of the background air. By mass, the majority of the metals in ambient air are of geologic origin and originate from fugitive dust or resuspension of paved road dust. Transition metals such as nickel and vanadium may exist at low concentrations as a result of industrial operations. Finally, background air consists of a mixture of gaseous pollutants such as ozone, NOx, SO_2, CO and hydrocarbons. The hydrocarbons are chemically diverse, and change in relation to atmospheric transformation.

Approach

The background atmospheres will include sulfate, nitrate, metals and gases. We propose a proportion of 40 per cent sulfate, 40 per cent nitrate and 20 % metals, with the total particulate concentration at 300 $\mu g/m^3$ as described above. When combined with motor vehicle emissions, the gas phase will be the same (hydrocarbons originate from MVE) but the mixed particle concentrations will be 200 $\mu g/m^3$ urban mix and 10 $\mu g/m^3$ MVE. The metal fraction will originate from resuspended road dust that was obtained from a high population density sample so it contains sufficient quantities of transition metals such as vanadium. The gas phase will match the gas phase that is found in the MVE, but will also include ozone that is maintained at 75 parts per billion concentration.

Sulfate: Sulfate aerosols will be generated with an evaporation-condensation approach. This approach uses nebulized sulfate (dilute sulfuric acid) that transits through a flow-through dryer to first remove water. Next the aerosol is heated to approximately 150°C and then recondensed in an aerosol line that is chilled through a heat exchanger operated at ~4°C. After further dilution, we have optimized the aerosol to produce a bimodal size distribution with sizes that are similar to ambient air. We will further dilute the aerosol and transit the material directly to a whole-body inhalation exposure chamber. In a sidestream, we will nebulize and dilute ammonia to a molar concentration that will provide approximately 70% neutralization of the sulfate when it reaches the exposure atmosphere. We will measure the extent of neutralization in the exposure atmospheres prior to exposures and, also once exposures are initiated, to ensure that the ammonia contribution from the animals is taken into account.

Nitrate: Our initial approach for the generating the nitrate exposure atmospheres will be to directly nebulize ammonium nitrate using an evaporation-condensation approach as described for sulfate. If this does not yield acceptable particle size, we also will evaluate the potential for using the evaporation-condensation approach starting with a dilute nitric acid solution and then combining the effluent with an excess of ammonia to react and form ammonium nitrate. Based on previous experience, these reactions occur rapidly within an inhalation chamber [(14)]. If necessary, we will add a reaction chamber to allow adequate time for the ammonium nitrate

reactions to occur. To ensure that there is no excess ammonia present, we will first pass the formed ammonium nitrate through a honeycomb denuder coated with citric acid that will bind and remove any excess ammonia in the mixture.

Metals/Road Dust: As mentioned above, metals in ambient air are primarily of geologic origin. Metals in the exposure atmosphere will be derived from road dust. In order to ensure that the metals contain transition metals such as nickel and vanadium are included, we will utilize paved road dust samples that we collected previously from heavily populated areas (Los Angeles, New York, New Jersey, Atlanta). Those samples, which are currently stored at -70°C, were shown to be composed primarily of geologic and carbonaceous material, but were significantly impacted with transition metals compared against samples collected from less populated areas [(14)]. We collected the dust from active traffic surfaces using a vacuum method. That material will be sieved on an orbital shaker and the finest fraction (<38 μm bulk material diameter) that has the highest chance of resuspension in the environment will be aerosolized. Aerosol will be generated using a Wright-Dust Feed (BGI Inc., Waltham, MA) coupled to a $PM_{2.5}$ cyclone to remove particles > 2.5 microns.

Gases: Gases for the background atmosphere will originate from MVE, with dilution to reduce concentrations to that are in proportion to particulate matter observed in ambient air. The exhaust will include hydrocarbons, CO, and small amounts of SO_2. Motor vehicle exhaust will pass through a HEPA filter to remove particulate matter. Ozone will be generated from medical-grade oxygen by electric arc using a calibrated ozone generator (OREC, Ozone Research & Equipment Corporation, Phoenix, AZ) and delivered to the airflow entering the chamber. O_3 will be introduced into the chamber and sampled using a Teledyne Instruments Ozone Monitor, Model 450 (Advanced Pollution Instrumentation, Inc., San Diego, CA).

Road Dust Atmospheres

Background and Approach: Road dust may be emitted in higher proportions from roadways than tailpipe emissions in some cases. Road dust represented a diverse composition of material that settles on the road through windblown dust, atmospheric deposition, tire and break wear, and mechanical breakdown of organics from vegetation. We previously collected and reported on the composition of road dust from locations throughout the United States, showing that road dust collected from more heavily populated areas do indeed have similar overall composition to less populated areas, but were also enriched in transition metals. We proposed to utilize previously collected and stored samples as described above. For the road dust atmospheres, however, the total concentration will be 300 $\mu g/m^3$, and when combined with motor vehicles the concentration will be 200 $\mu g/m^3$ road dust and 100 $\mu g/m^3$ MVE.

Aim 2: Conduct inhalation exposures of laboratory animals. Aim 2 will directly support the animal toxicology project. The results of the animal toxicology studies will also inform this project by providing feedback for iteratively testing the hypotheses and for selecting or modifying exposures for humans and subsequent animal studies focusing on specific biological responses. The first round of inhalation exposures will generate substantial biological data and important information on the relative ranking and potential interactive effects of roadway emissions. The subsequent exposures of humans will determine comparability across species, and subsequent exposures of animals will refine our understanding of the biological mechanisms involved and point toward specific causal pollutants. Methods for statistical analysis of

biological response versus exposures are described in the animal and human toxicology projects, and in the Biostatistics Core.

System Validation and Conduct of Exposure: Once an exposure system has been developed, the conduct of the study includes: development of standard operating procedures (SOPs) for system operation and safety, pre-study exposure system validation; training and training certification of system operators; and conduct of exposures. SOPs document the conduct of all aspects of the study and the step-by-step procedures for operating the generation system, collecting environmental or exposure monitoring data, and safety. Institutional SOPs are reviewed and approved by LRRI management, and study-specific SOPs are approved by the Principal Investigator who oversees each function. Once an SOP is developed, each technician participating in that portion of the study is trained, and training is documented.

Characterization of Inhalation Exposure Atmospheres: The characterization of the exposure atmospheres will mirror characterization of ambient exposures in Project 1, and will also extend to include the major chemical components that are measured in the National Chemical Speciation Monitoring Sites. The effort will be a direct partnership between Projects 1 and 2. Table 2 summarizes the analyses to be conducted on laboratory inhalation atmospheres. As described further in Project 1, the aerosol time-of flight aerosol mass spectrometer will be able to define chemical formation of SOA in the laboratory (and ambient) atmospheres. This, combined with the analysis of changing volatile inorganic and organic gases, will build understanding on the composition and transformation of motor vehicle emissions in both ambient air and the laboratory. Descriptions on methodology for the characterization methods for the time of flight aerosol mass spectrometer and proton transfer reaction mass spectrometer are included in Project 1. Details on methodology for additional measurements are previously described [5].

Table 2. Summary of PM and co-pollutant properties that will be utilized to characterize exposure atmospheres

PM/Co-pollutant Properties	Method of Characterization
Physical Characteristics	
Size (coarse, fine, ultrafine)	Impactor, differential mobility
Number	Condensation particle counter
Surface area	Differential mobility/calculations
$PM_{2.5}$ mass concentration	Gravimetry
Chemical Characteristics	
Total metals and elements	X-Ray fluorescence
Carbon	
Black carbon (elemental carbon)	Thermal/optical analysis
Organic carbon	Thermal/optical analysis
Organic carbon class/species	GC/MS and LC/MS/MS
Particle Organic Composition	Aerosol MS
Ammonium	Colorimetry
Sulfate/nitrate compounds	Ion chromatography
Co-Pollutants	

Nitrogen oxides	Chemiluminescence
Ozone	UV absorption
Gas phase hydrocarbons	
Total hydrocarbons	Flame ionization
Speciation of gas hydrocarbons	Proton Transfer Reaction MS
Carbon monoxide	Infrared
Sulfur dioxide	Sorbent/ion chromatography
Ammonia	Sorbent/ion chromatography

Abbreviations: GC: gas chromatography; MS: mass spectrometry; LC: liquid chromatography; PCR: polymerase chain reaction; UV: ultraviolet

Exposure Concentrations: As mentioned above, the target exposure atmospheres will be normalized to a maximum particle concentration of 300 $\mu g/m^3$. When investigating combination atmospheres, the urban background or road dust will be combined at 200 with 100 of the MVE, which is one of the concentrations of MVE proposed for the dose-response studies. Animals will be exposed 6 hr/day for 7 and 50 days. For the studies comparing fresh versus aged exhaust, a treatment group will also be included that provides matching particle number concentrations. The concentrations of gaseous components will vary by exposure atmosphere, but will be targeted at 3-5 times what is typical in ambient air, similar to the enrichment of particulate matter that is proposed. This will include ~0.5-1 ppm for NOx, 1-5 ppm for CO, 0.05 ppm SO_2 and 0.2 ppm for hydrocarbons. When ozone is included it will be present at a concentration of 1.5 X the current ambient standard of 75 ppb.

Dilution and Exposure Systems: We dilute with tempered (~15-20°C) and charcoal- (to remove volatile contaminants) plus HEPA-filtered (to remove PM) ambient air. Although the specific approach for dilution will depend on the PM source and the requirements of the exposure, the general approach will follow the strategy and recommendations that have been described by [12] for controlled engine emission sampling (aimed at achieving representative particle sizes for combustion emissions). The general approach will be to dilute PM immediately after combustion or aerosol generation to ensure that particle nucleation events are quenched as rapidly and to the greatest extent possible. The maximum dilution will be limited by the desired exposure concentration. For engine exhaust, an in-line flow-through muffler will be used on each exposure line to reduce exposure chamber noise levels to less than 85 dB.

Rodent Exposure Chambers: Exposures will be conducted in custom made 5L whole-body rodent inhalation chambers. The chambers will house up to 40 mice at a time. The chambers will be ventilated with exposure atmospheres at approximately 10 L/minute, yielding a residence time within the chamber of about 30 seconds. These chambers are designed to enhance the uniformity of the aerosol distribution throughout the chamber during exposure [15,16; 5]. The chambers contain sampling ports above each cage unit to facilitate characterizing spatial homogeneity of exposures and to provide multiple sample locations for exposure characterization.

Aim 3: Conduct inhalation exposures of human subjects. Informed by the results of Aims 1 and 2, exposure scenarios will be selected for the human studies. As described above, the animal toxicology studies will inform the selection of conditions for the experimental human atmospheres. Although the selection cannot be known at this time, we envision conducting exposures to MVE with and without ozone, and exposures to the atmospheres proved most and least potent in the animal experiments. Thus, we envision evaluating the effects of four atmospheres in the human exposure studies.

The human studies are described in Project 4. After the initial screening phase of the animal experiments are concluded, the relative potency of the atmospheres will be assessed. The selected exposure technology will be transferred to the human exposure lab. The UW human exposure lab is an existing infrastructure that has been conducting diesel inhalation exposure studies since 2003. A critical component of Aim 3 will be the transfer and optimization of the exposure atmosphere at the UW laboratory. Because this Aim will be informed by results yet to be collected, the specific approach cannot be defined at this time. It is important, however, to define the process to ensure that the generation technology and thus the exposure atmospheres are appropriately transferred.

Transfer of Exposure Atmosphere Technology to UW: While the human exposure atmospheres remain to be defined, we can be certain that the minimum requirement will be the mixed motor vehicle emissions (MVE) exposure atmosphere. The UW diesel exposures are already similar in composition to the LRRI animal exposures because the generation and dilution system were based on LRRI experience [5]. We will also transfer the technology for generating gasoline exhaust as it is done at LRRI, and also the methodology for mixing diesel and gasoline exhausts.

The transfer of atmospheres of road dust, ozone, background air, and atmospheric aging are all challenging but logistically straightforward, considering the experience of the proposed Project team in designing and building these types of exposure systems. The most technically challenging transfer of technology (if required) will be the irradiation chamber. A space for the chamber at the UW facility has been identified. LRRI has built two such chamber systems, and has designed a cost-effective approach for constructing a chamber at the human exposure laboratory having capabilities adequate for these studies. Specifically, an internal frame will be built out of uni-strut. The metal frame will be surrounded by a specially cut Teflon bag with penetrations for introduction of reactants and extraction of transformed products. The bag will be maintained in a plywood enclosure that is surrounded with ultra-violet lights. Because of the expected reactivity of the mixture, the number of lights required for chemical transformation are modest, and challenges such as excessive temperatures are not anticipated.

Expected Results/Benefits: This project will help elucidate the important characteristics that define toxicity resulting from roadway emissions and their interaction with background air. We expect that fresh whole exhaust containing ultrafine particles and vapor will confer the most potent atmosphere. These results will be confirmed in both rodent and human studies. Additional results and benefits will be the development of new exposure techniques to manipulate exposure atmosphere composition of complex combustion emissions through physical and chemical transformation. The chemical transformation of MVE, especially gasoline engine emissions is very poorly studied. This project will help define the composition of atmospherically transformed MVE, and the potential for MVE to form secondary organic aerosol.

GENERAL PROJECT INFORMATION

Organization and Management: The Project Principal Investigator, Dr. Jacob D. McDonald, will manage the technical work of developing exposure atmospheres and conducting exposures. He will have the primary responsibility for documenting, analyzing, interpreting, and reporting results to collaborators and Center Director. Dr. McDonald serves as Director of the Chemistry and Inhalation Exposure Program at LRRI. This group includes a staff of approximately 15 engineers/technicians and 10 chemists with decades of experience in the development and characterization of laboratory exposure systems. Dr. McDonald and his team have extensive experience in the development of complex exposure atmospheres for laboratory studies, and in integrating these systems with multidisciplinary research.

Co-Investigators on Project 2 include Drs. Joe Mauderly, Melanie Doyle-Eisele and Tim Larson. Dr. Mauderly has several decades of experience in air pollution research, and in managing multidisciplinary studies to assess the potential toxicity of complex exposure mixtures. Dr. Mauderly will serve as an unpaid advisor to the project. Dr. Doyle-Eisele is an atmospheric chemist and toxicologist. She received her PhD from the University of North Carolina in 2005 with a focus in toxicology of atmospheric transformation of gases in smog chambers. Dr. Doyle-Eisele will work with Dr. McDonald to develop the smog chamber atmospheres. Dr. Larson is currently a technical lead on the University of Washington human exposure facility. Drs McDonald and Larson will work directly together to ensure a transition of the exposure atmospheres and technology at LRRI to UW for the human exposure experiments.

Schedule and Timeline: The first 3 years of Project 2 is focused on animal exposure studies (Project 3), which will transition to a focus on new exposure atmospheres to support human exposure studies (Project 4) with novel exposure atmospheres during years 3-5. Years 1-2 will include the conduct of inhalation exposures and the development of novel exposure atmospheres. Subsequent years will include further animal studies to confirm initial results and study mechanism of toxicity. The human studies will be informed by the results of the animal studies, such that the exposure atmosphere generation technology for a limited set of atmospheres will be transferred to the human exposure laboratory.

Facilities, Infrastructure, and Capabilities: The animal exposures will be conducted in the 300,000-sq.-ft. LRRI Inhalation Toxicology Laboratory (ITL, formerly Inhalation Toxicology Research Institute), which has a long-standing history of excellence in the generation and characterization of controlled inhalation exposures of animals, including simple and complex atmospheres of environmental relevance. The MVE, irradiation chamber and road dust inhalation systems are already in place for this work. Those facilities have been developed as part of existing programs under the auspices of the National Environmental Respiratory Center (www.nercenter.org), the Health Effects Institute NPACT program, and the Electric Power Research Institutes SPHERE program. Only modest modifications to the existing infrastructure are required for this work. LRRI's Animal Care Unit and animal facilities are integral to the ITL, and the staff is accustomed to close coordination of animal care and exposure operations.

The University of Washington human exposure laboratory was established in 2003 Since that time, approximately 150 exposure sessions each for humans and animals have been conducted, including with diesel engine emissions as previously described [17]. Details regarding the human exposure laboratory are provided in Project 4.

PROJECT 3: Cardiovascular Consequences of Immune Modification by Traffic-Related Emissions

OBJECTIVES

Traffic-related emissions are associated with the incidence and progression of acute and chronic cardiovascular sequelae in human population studies. Such phenomena of near-roadway health effects have yet to be characterized toxicologically. Because of overlapping issues related to noise, socioeconomic status, ethnicity, etc, there is a need to better understand the biological plausibility that fresh mixtures of vehicular emissions have a focused impact on human health. The complexity of the pollutant mixture, with particles and gases, fresh and aged, makes it difficult, if not impossible, to unravel the relationships with epidemiological approaches.

We have observed that fresh, whole emissions can drive the oxidation of lipids and inflammation in atherosclerotic regions, an effect which is dependent on both particulate and gaseous components of the emissions. Furthermore, the combination of fresh gasoline engine emissions, which are relatively low in PM mass and high in volatile organic compounds, with higher PM-containing diesel engine emissions led to synergistic increases in vascular lipid peroxidation. Additionally, while numerous toxicology studies indicate a promotion of atherosclerosis in animal models, the mechanisms underlying this phenomenon remain uncertain. Contemporary concepts in vascular disease suggest a vital role for lipid-induced activation of the immune system. More specifically, oxidative transformations of endogenous phospholipids can promote vessel disease through 1) interactions with specific immune-modulating receptors (e.g., TLR-4, CD36, LOX-1) and also 2) through recognition by innate antibodies.

We have also found that blockade of a surface receptor for oxidized lipids (LOX-1) can reverse the vascular oxidative stress from vehicular emissions exposures. Other related receptors, including CD36 and TLR-4, may also interact with specifically-modified phospholipids. *We hypothesize that emissions-induced oxidation of certain endogenous phospholipids, presumably in the pulmonary surfactant, can stimulate the activity of immune cells through such receptors and in turn promote the monocytic invasion of existing vascular lesions*. We will test this hypothesis concomitantly with investigations as to the relative potency of roadway-related pollutants, including mixed vehicular emissions and road dust.

Thus, in **Aim 1**, we will ascertain the potentiating effects of physical and photochemical aging on fresh emissions, in terms of driving this vascular oxidative stress**.** Combined diesel and gasoline emissions will be modified by physical and photochemical aging, and also produced in combination with resuspended road dust and also a modeled urban background. Importantly, this Aim will be vital to providing information to Projects 2 and 4 on the most potent atmosphere to be used for human exposure studies.

In Aim 2, we will examine effects of the emissions-induced oxidative modifications to endogenous phospholipids, in terms of activating immune-modulating receptors such as LOX-1, CD-36, TLR-2, and TLR-4. This Aim will utilize receptor-deficient mouse models to examine the roles of these receptors, as well as characterize the lipidomic alterations in various tissues.

Lastly, **in Aim 3**, we will further explore the role of specific immune cell populations as participants in the innate and adaptive responses to emissions-induced phospholipid modifications. In this Aim, we will utilize a mouse model of immunodeficiency, the severe combined immune deficiency (SCID) mouse. Additionally, we will pursue bone-marrow transplants from mice lacking those receptors described in Aim 2 into SCID mice to mechanistically establish the involvement of the oxidatively-modified phospholipids.

BACKGROUND

Atherosclerosis and Air Pollution: Public Health Significance

There is little doubt that atherosclerosis, as the etiological basic for coronary artery disease, ischemic heart disease, and other adverse cardiovascular sequelae, represents one of the most significant health problems in the developed world.[5] According to the American Heart Association, more then 70 million (2003 statistics) Americans are living with at least one form of cardiovascular disease. While several genetic, behavioral, and dietary factors clearly drive the disease, air pollution remains a potential contributor to which all people are exposed, no matter their lifestyle or genetic background.

The epidemiological association between overall cardiopulmonary morbidity and mortality has been known since the publication of the Six Cities Study.[10] Since then, numerous studies have indicated that cardiovascular disease drove such associations.[36] Newly emerging evidence from epidemiological research suggests that source-specific air pollution may have a focused impact on cardiovascular health. In particular, exposure to traffic has been shown in some studies to be a stronger risk for acute myocardial infarction, and the proximity to roadways was better associated with coronary artery calcification than were measures of particulate matter (PM) exposure.[16,17,34] Thus, the strong statistical signal observed in these studies may reflect a cumulative impact of the components of fresh vehicular emissions, rather than toxicity of individual pollutants.

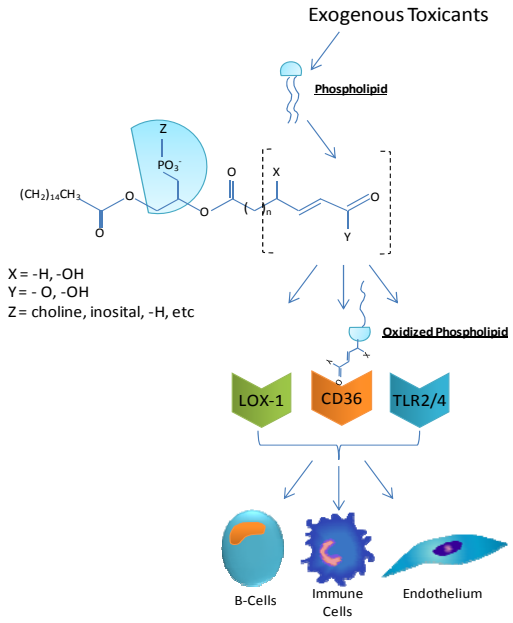


Figure 1. Hypothesized activation of inflammatory pathways in atheroclerosis. The fatty acid tail of a phospholipid is oxidized, leading to a number of varying chemicals that have enhanced ligand recognition for the scavenger receptors. Additionally, innate antibodies recognize modified phospholipids, reflecting the triggering T and B cell-dependent pathways.

Receptor-Mediated Activation of Immune Cells by Oxidatively Modified Phospholipids

The overriding hypothesis for the current proposal is that oxidized lipids, originating from exposure to environmental pollutants, can interact with macrophages and T cells, and thereby influence plaque inflammation (Fig 1). Numerous studies have investigated the role of oxidation products of a specific phospholipid component of oxLDL, 1-palmitoyl-2-arachidonyl-sn-glycerol-3-phosphocholine (PAPC), in driving vascular inflammation in atherosclerosis models[13,41]. Oxidized PAPC can effectively bind the CD36 receptor and compete with oxLDL[4].

Several toxicological studies have now linked systemic vascular effects of air pollution with activation of the innate immune system.[7,33,40] The association between oxidized phospholipids and specific immune-modulating receptors (e.g., CD36, LOX-1, TLR2/4) is being elucidated in various models of cardiovascular disease. LOX-1 has been shown to be crucial to the oxLDL-induced upregulation of metalloproteinases in endothelial cells[24] and LOX-1 blockade can reverse the endothelial dysfunction associated with hyperlipidemia in mouse models.[42] Moreover, LOX-1 has been shown to be increased in vulnerable regions of atherosclerotic plaques where macrophage infiltration is high due to apoptosis/necrosis.[20] Likewise, the Toll-

like receptor (TLR)-2 has been shown to have a vital role in potentiating vascular lesions in mouse models[32]. Disruption of TLR signaling by selectively removing the intermediate MyD88 molecule led to significant reductions in vascular recruitment of macrophages and MCP-1 levels.[3]

In atherosclerosis, the CD36 receptor interacts with oxidized lipids resulting in signals that promote the pathology.[22] CD36 expression is regulated by various nuclear receptors, including PPARγ, Nrf2, and FoxO1[2,19]. While these receptors have a role in handling dietary lipids, oxidized lipids seem to have a more prominent effect on this pathway, and potentially drive air pollution-induced plaque promotion.

Mouse Models of Atherosclerosis and Air Pollution

Several mouse models of atherosclerosis, including the apoE$^{-/-}$ and LDLR$^{-/-}$, are susceptible to vasculotoxic effects of air pollutants, both acutely[1,6] and chronically.[7,25,39] When apoE (or the LDL receptor) is deleted from the genome, mice develop severely elevated lipid and cholesterol profiles; apoE$^{-/-}$ mice on a high-fat ("Western") diet exhibit cholesterol levels exceeding 1000 mg/dl (normal is ~150 mg/dl[18]). As a result, the lipid uptake into the vasculature is increased and the atherosclerotic process is dramatically hastened. Furthermore, LDL isolated from apoE$^{-/-}$ mice is highly susceptible to oxidation,[15] which we postulate is a crucial event in the air pollution-mediated vascular changes.

We will use this sensitive hypercholesterolemic model as a platform for testing the roles of specific immunomodulatory receptors and immune cell pathways in driving the vascular effects of complex emissions. As several studies have noted activation of inflammatory pathways in an associative manner following exposure to particulate matter,[7,33,40] the mechanistic design of the present study will be invaluable to our understanding of the health impact of air pollutants.

PRELIMINARY DATA

The following section details the findings from a variety of studies and exposures conducted within the framework of the National Environmental Respiratory Center (NERC) or the National Particle Component Toxicity (NPACT) Initiative. The exposure paradigm includes whole body inhalation exposures for 6 h/d for either 7 or 50 days and the apoE$^{-/-}$ mouse model is the most frequently used. Thus, studies between gasoline, diesel, coal, and woodsmoke combustion, and mixtures thereof, are directly comparable in terms of animal model and exposure conditions.

Table 1 shows components from the highest level of gasoline engine emissions and the medium level of diesel, hardwood, and coal combustion used for these studies. For mixed vehicular emissions (MVE), diesel and gasoline were mixed such that roughly 250 μg PM/m^3 was derived from diesel and 50 μg PM/m^3 was derived from gasoline.

Table 1. Basic Characterization of Combustion Atmosphere Composition

	Particle Mass μg/m^3	Carbon Monoxide ppm	Nitrogen Monoxide ppm	Nitrogen Dioxide ppm	Total Hydrocarbons mg/m^3
Gasoline Exhaust	59	104	16.7	1.1	15.9
Diesel Engine Exhaust	320	10.2	18	0.9	0.6
Hardwood Smoke	320	4.0	0	0	1.4
Simulated Coal Combustion	313	0.04	0.2	0.1	<0.1
Mixed Vehicular Emissions	306	102	14	1	3.7

Pollutant Exposures Alter Phospholipid Structure in Erythrocytes

Oxidation products of PAPC have known roles in promoting atherosclerosis.[23,27] In order to measure these and other phospholipid by-products in biological samples, we collected

erythrocytes from apoE$^{-/-}$ mice exposed to coal emissions (control, 100, or 1000 µg PM/m^3 for 50 days) and conducted lipidomic analysis with colleagues at the University of Georgia (Brian Cummings, Ph.D.). Here, the phospholipids were generally just components of the plasma membrane, and not specifically related to adipocytes or LDL. We quantified the concentrations of specific m/z ratio peaks by LCMS and found a great number of significantly modified entities. More importantly, as shown in figure 2a, it was clear that a dose-dependent formation or destruction was occurring for *every phospholipid compound*, although not always statistically significant. We also looked at a specific component of PAPC oxidation, POVPC, and found that it was roughly 3-4 fold increased by the coal exposures (Fig 2b). Strong evidence supports a role for POVPC in atherosclerosis progression and inflammatory cell activation.[35] It is interesting to note that these modified phospholipids were found 1) in the circulation and 2) in a model of exposure (coal) that was modest, at best, in terms of vascular pathology.

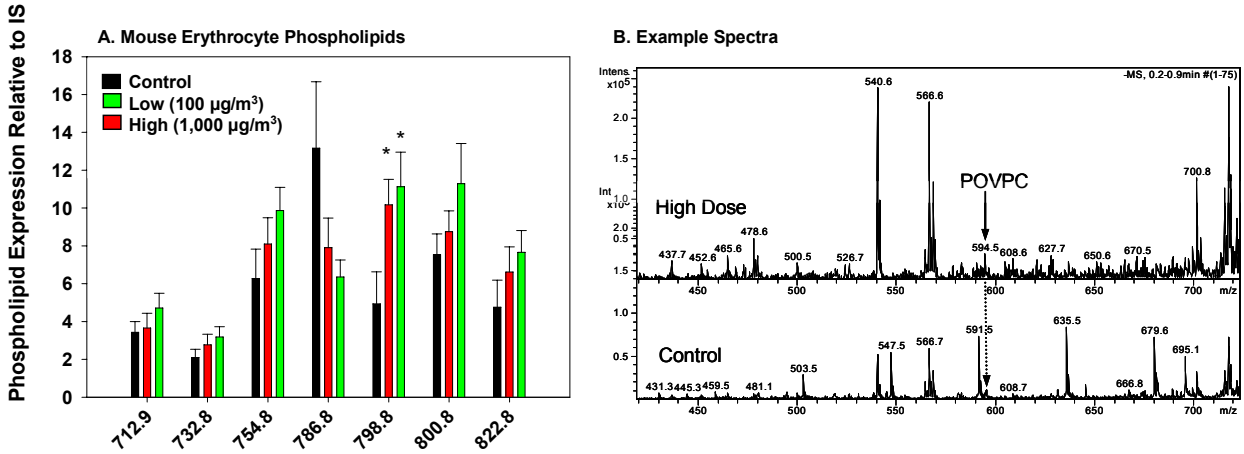


Figure 2. Concentration-dependent modification of erythrocyte phospholipids. A) Quantitative comparison of 7 consecutive phospholipid species (m/z ratios from 712.9 to 822.8; much more data were collected than shown here) for coal emissions-exposed mice. ApoE$^{-/-}$ mice (n=5/gp) were exposed for 50 days to filtered air (Control), low (100 µg PM/m^3), or high (1,000 µg PM/m^3) levels of simulated downwind coal emissions, relative to an internal standard (IS). Concentration-related changes of increasing or decreasing nature were revealed for most principal spectral peaks. B. A specific oxidatively-modified phospholipid, POVPC (at 594), the primary oxidation product of PAPC and known contributor to atherogenesis, was increased, albeit a minor component of the overall erythrocyte lipid population.

Ozone and MVE Induce Vascular LOX-1 Receptors

Among the more interesting findings was that the receptor responsible for scavenging of oxLDL, LOX-1, is selectively upregulated in vascular tissue following exposure to certain pollutants. In a recent study, our collaborators at the EPA (U. Kodavanti, PI) exposed male Wistar Kyoto rats (12-15 wk old), nose-only to air, ozone (O_3; 0.5 ppm), re-

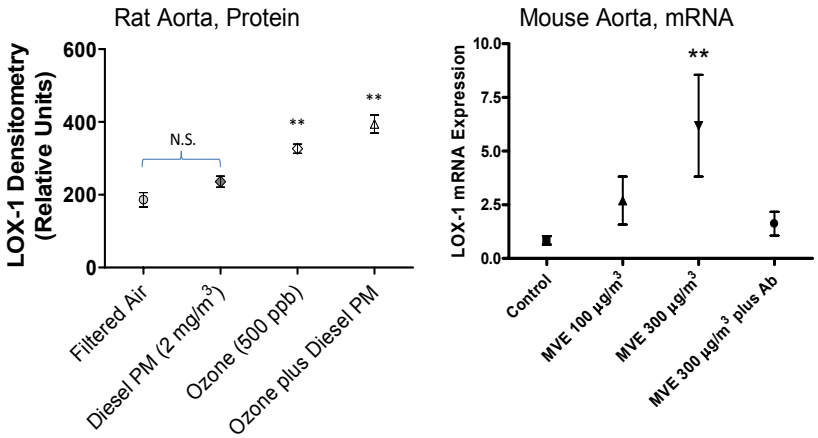


Figure 3. *Left*, LOX-1 protein measured in aortas from Wistar Kyoto rats exposed to diesel PM and/ or ozone for 16 weeks. *Right*, LOX-1 mRNA from ApoE$^{-/-}$ mouse aortas exposed for 7 days to mixed vehicular emissions (MVE). Asterisks (**) indicate significant difference from control ($p<0.01$, by ANOVA with Newman-Kuels posthoc comparison).

suspended diesel exhaust particles (DEP) from a 30 kW Deutz engine (2.0 mg/m^3), or DEP plus O_3, 5 h/d for 1 d/wk for 16 wk. As expected by these levels of pollutants, modest pulmonary inflammation was evident in both O_3 and/or DEP rats. We found that aortic LOX-1 protein was significantly upregulated by ozone (500 ppb) and ozone plus diesel PM (500 ppb and 2 mg/m^3), but not PM alone (Fig 3). While it was perhaps surprising that PM had no significant effect, we suspect that the ozone had a specific effect on the surfactant, leading to modification of bioactive phospholipids.

In apoE$^{-/-}$ mice, we have also seen a significant upregulation of aortic LOX-1 mRNA following exposure to the mixed diesel and gasoline emissions (MVE; Fig 3). Mice were exposed to filtered air or two concentrations of MVE, 100 or 300 µg/m^3 daily for a week. A separate group was administered a neutralizing antibody to LOX-1 (ip injection) every other day for the study. Exposures caused a significant concentration-dependent increase in LOX-1 mRNA, and neutralizing antibody treatment reduced this effect.

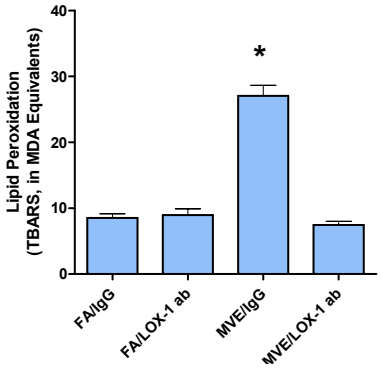


Figure 4. After 7 days of exposure to mixed vehicular emissions (MVE; 300 µg PM/m^3), aortic TBARS were significantly elevated compared to groups exposed to filtered air. Treatment with the antibody (ab) to the LOX-1 receptor blocked this effect.

LOX-1 Drives Systemic Vascular Effects

Following the findings of increased vascular LOX-1, we co-treated apoE$^{-/-}$ mice with a neutralizing antibody against this LOX-1 scavenger receptor concomitantly with exposures. Thus, for these LOX-1 inhibition studies, mice were dosed with either nonimmune mouse IgG (16 µg protein/ml, 0.1 ml/mouse; ip) or the neutralizing antibody to LOX-1 (4 doses of anti-mouse LOX-1/SR-E1 antibody: 16 µg protein/ml, 0.1 ml/mouse; ip; R&D Systems) for the week of exposures (every other day, beginning the afternoon before the first exposure). Each group included 8 mice and they were exposed for 6 h/d for 7 days. The groups included control (filtered air)+IgG, control+anti-LOX-1, MVE-exposed+IgG, and MVE-exposed+anti-LOX-1.

Our results indicate that the LOX-1 receptor might be central to the genesis of lipid peroxides. We found that MVE induced a significant increase in lipid peroxides that was completely blocked by the LOX-1 antibody treatment (Fig 4). This treatment was similarly effective in reducing other reported vascular responses, including MMP-9 and ET-1.[25]

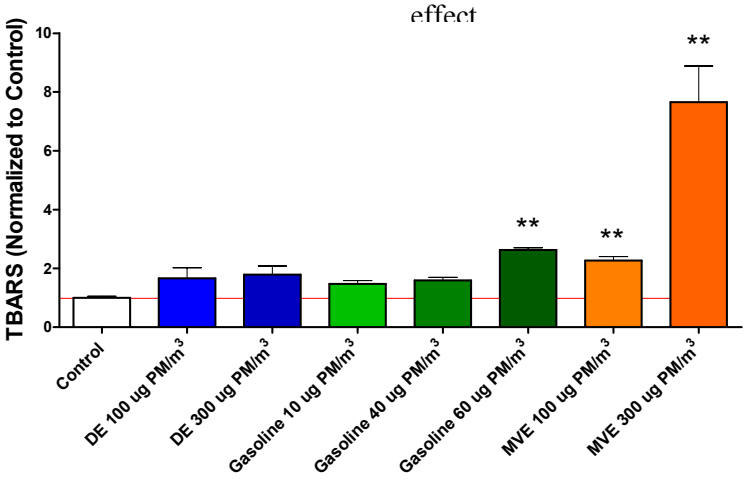


Figure 5. Comparison of lipid peroxidation effects from diesel, gasoline, and combined (MVE) emissions at various concentrations. Aortas were obtained from apoE-/- mice following 50 days of exposure. Asterisks indicate significant difference from control (P<0.01 by ANOVA with Bonferroni Posthoc Comparison).

Combined Gasoline and Diesel Synergistically Enhance Vascular Lipid Peroxidation

While we have found significant increases in aortic lipid peroxidation following gasoline[25,26] and diesel[7] independently, we decided to examine this endpoint in a "urban" model of mixed vehicular emissions. To accomplish this, we combined a portion of gasoline emissions containing 50 μg PM/m^3 with a portion of diesel emissions containing 250 μg PM/m^3 and exposed $apoE^{-/-}$ mice for 6 h/d x 50 days.

In those previous studies, we observed a relative increase above control of between 2-3 fold for both gasoline (60 μg PM/m^3) and diesel (300 μg PM/m^3) emissions. When the combination of slightly less than these concentrations was studied, we achieved a >7-fold increase over control (Fig 5). At a lower concentration of MVE (100 μg PM/m^3; same dilution ratios), we also saw a dramatic increase compared with similarly lower concentrations of the individual emissions.

MVE Gases plus "Background" Secondary PM Potentiates Aortic Lipid Peroxidation

Because we found a significant enhancement with the combination of gasoline and diesel emissions, we investigated the extent to which this was a PM-dependent toxicity. We have previously found that removing PM from the high concentration of gasoline emissions (60 μg/m^3) did little to the lipid peroxidation response, while removing PM from diesel had a more compelling, albeit not statistically distinguishable effect.[7] However, with the combination of gasoline (high VOCs) and diesel (high PM), we induced such a dramatically increased lipid peroxidation, we were not surprised that removal of the PM reduced the relative level of lipid peroxides to nearly the level of controls, although some effect was observed (~1.5x control compared to the >7x control when PM was present; Fig 6).

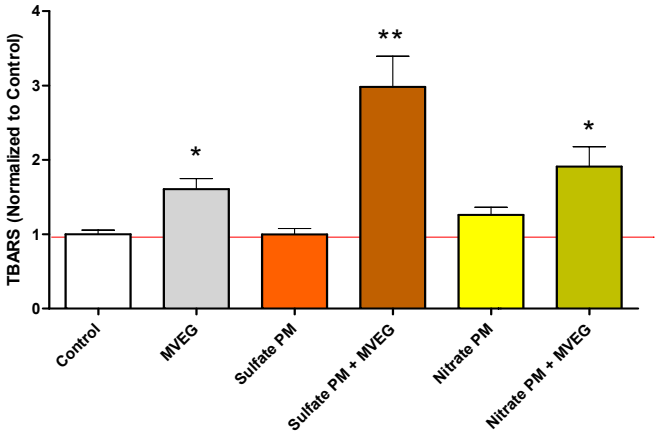


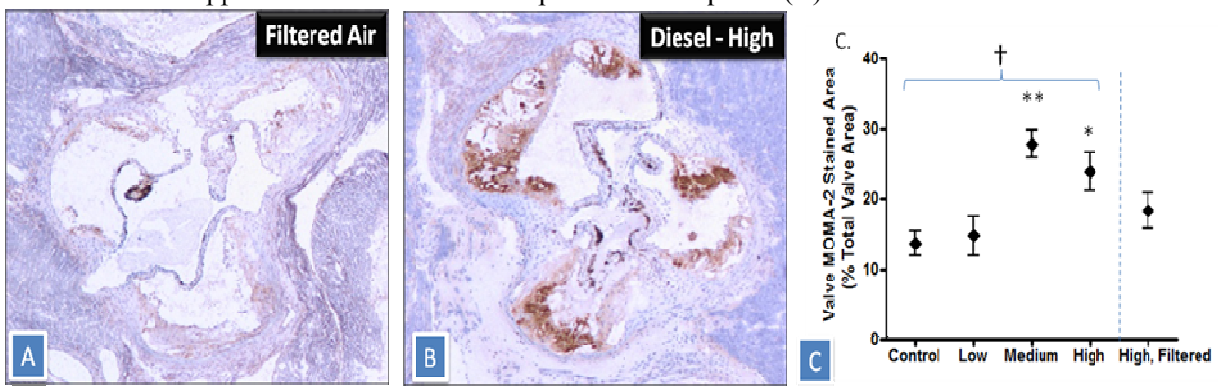
Figure 6. Removal of PM from MVE (MVEG) caused a dramatic reduction in the levels of aortic TBARS (Figure 5). However, when sulfate or nitrate PM (at 300 μg/m^3) was added to MVEG, a significant increase in lipid peroxidation was observed (*,$P<0.05$ and **,$P<0.01$; by ANOVA).

Because removing PM dramatically attenuated the lipid peroxidation capacity of MVE, we then decided to examine whether other sources of PM, such as might be present in background conditions, might also synergize with the vapor phase pollutants. To this end, an MVEG atmosphere was combined with secondary sulfate or nitrate PM and $apoE^{-/-}$ mice were exposed by whole body inhalation for 6 h/d x 50d, as above. Lipid peroxidation, as measured by the TBARS assay, was again found to be significantly elevated by combined PM and vapor phase components, but not by the secondary PM alone.

Diesel Induces Vascular Macrophage Infiltration

In most of our earlier studies, we did not examine the histological ramifications of pollutant exposure. However, with the aortic leaflet region from $apoE^{-/-}$ mice exposed to diesel exhaust at 0, 100, 300, or 1000 μg/m^3, we stained cryosections for a marker of macrophages, MOMA-2. We observed a significant increase in macrophage infiltration at the 300 and 1000 μg/m^3 levels (Fig 7). This effect was significantly attenuated by PM filtration, similar to aortic TBARS.

Figure 7. Images of aortic leaflet region staining for plaque compositional changes due to diesel emissions exposure. ApoE$^{-/-}$ mice were exposed to whole diesel engine emissions for 50 days at 0, 100, 300, and 1000 µg PM/m^3 or a PM-filtered atmosphere (with gaseous components at the levels in the highest exposure). Macrophage staining (MOMA-2; dark brown) was relatively light in control mice (A), while vascular lesions from mice exposed to diesel engine emissions exhibited substantial macrophage infiltration (B). Overall, significant recruitment was observed at the 300 and 1000 µg PM/m^3 levels and filtration of PM appeared to abolish at least a part of this response (C).



Negative Findings: What We Know Does NOT Drive Lipid Peroxidation

In the pollutant atmospheres examined, carbon monoxide (CO) and oxides of nitrogen (NO and NO_2) were often outside of the range of allowable limits (Table 1). We conducted head-to-head 7-day exposures to all complex atmospheres along with these gases independently to ascertain whether they could drive the lipid peroxidation. Thus, we exposed apoE$^{-/-}$ mice to gasoline, diesel, hardwood, coal, or mixed emissions or 80 ppm CO, 17 ppm NO, or 2 ppm NO_2 for 6 h/d x 7 days. Gasoline and diesel induced significant aortic lipid peroxidation, while hardwood smoke and the simulated coal atmosphere had no effect (Fig 8). Combined gasoline and diesel emissions at ~300 µg PM/m^3 caused additional peroxidation, though not synergistically as observed previously (Fig 5). The principal gases CO and NOx were not responsible for increases in aortic lipid peroxidation. Combined with earlier results of secondary PM (Fig 6), in the absence of gases, we begin to see the importance of the volatile organic portion of the complex combustion atmospheres, and the interaction with co-pollutant particles.

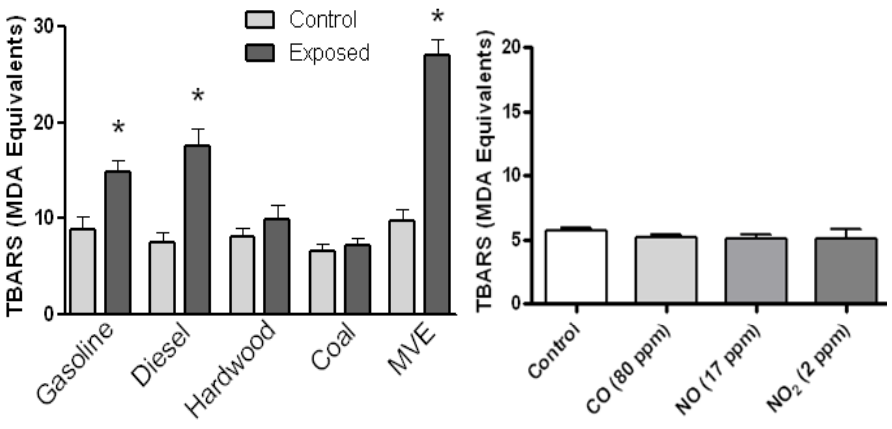


Figure 8. Assessment of 7-day exposure effects on lipid peroxidation (TBARS) in ApoE$^{-/-}$ mice exposed to various complex combustion atmospheres. Asterisks indicate significant difference from control ($p<0.05$; N=8-10/group). TBARS levels were unaffected by exposure to gases at the levels observed in gasoline emission studies.

APPROACH/ACTIVITIES

Specific Aim 1. We will ascertain the potentiating effects of physical and photochemical aging on fresh emissions, along with interactions with background material and road dust, in terms of driving this vascular oxidative stress**.** To accomplish this, we will link our project with Project 2, led by Jacob McDonald, Ph.D., wherein sophisticated and innovative exposure atmospheres will be generated and validated with real-world measurements of roadway and near roadway conditions. *These studies will provide information as to the most adverse transformational scenarios related to the inhalation of traffic-related pollutants, which will help refine approaches used in Project 4 and also future research at LRRI and elsewhere.*

Rationale: Proximity to roadways and, ostensibly, traffic-related emissions have been recently reported to be a driver of acute and chronic cardiovascular sequelae. This epidemiologically-observed phenomenon of near-roadway health effects has yet to be characterized toxicologically.

Numerous dynamics impact the chemistry of traffic emissions, including the relative mixture of gasoline and diesel emissions, dilution effects, physical aging, and transformative photochemistry. All of these can be tested in a controlled setting using realistic exposures and sensitive biomarkers of vascular toxicity. In Project 2, our colleagues will generate a mixture of gasoline and diesel engine emissions and test the transformations incurred by 1) physical aging and 2) photochemical aging.

The present Aim will incorporate these permutations, along with the factors of dilution/concentration and ratio of diesel:gasoline contributions to determine the most potent conditions for vascular lipid peroxidation. Importantly, the results of these studies will provide direction to the clinical studies of Project 4. The projected exposure conditions/atmospheres are described in Table 2, with greater detail as to the concentrations and procedures in Project 2.

Animal Model Selection: For the first exposures (diesel: gasoline ratio studies), we will test 4 groups of mice: apoE$^{-/-}$ mice on a normal chow, apoE$^{-/-}$ mice on a high fat chow, LDLR$^{-/-}$ mice on a regular chow, and LDLR$^{-/-}$ mice on a high fat chow. The LDLR$^{-/-}$ model will help assess the universality of the vascular response, and the regular and high fat chow permutations will allow us to determine the role of hypercholesterolemia. We will test 10 animals per group to enhance our ability to compare the present exposure with those to follow. To balance the atherosclerotic models, we will begin apoE$^{-/-}$ mice at 10 weeks of age and LDLR$^{-/-}$ mice at 16 weeks of age.

Screening Process: Ultimately, Projects 2, 3, and 4 will be highly interactive in the sense that results from each study will help direct either the exposure scenarios or biological assays of each other. First, with the comparison of diesel:gasoline ratios, we will determine the most toxic combination for use in the physical and photochemical aging scenarios. Additionally, this will help direct exposures in the latter years of this project and in Project 4. Similarly, assessing the roles of physical and photochemical aging will provide guidance. We assume that both will have detoxifying effects, but acknowledge the possibility that either could lead to unpredicted increases in toxicity. Lastly, the combination of MVE with road dust and the modeled urban background with further elucidate potential interactions of fresh emissions with related copollutants.

Endpoints: To provide the most robust comparisons between atmospheres, our priority endpoint for these studies will be lipid peroxidation in the lungs and aortas, as measured by the TBARS assay. While non-specific, this assay provides us with the most sensitive and stable information for comparison studies. Secondary endpoints will include 1) histopathology on the lungs and aortic sinus region, to assess infiltration of macrophages and other immune cells, as well as 2) levels of 4-hydroxynonenal, a well-defined by-product of lipid peroxidation.[38]

Table 2. Summary of exposures for Aim 1.

Atmosphere	Description	Control N	Exposed N	Duration
A. MVE	Test Ratios of gasoline:diesel at 300 µg PM/m^3. As we have observed that a diesel:gasoline PM ratio of 5:1 is synergistically toxic compared to either gas alone, we will generate 4:1, 9:1, and 14:1 PM ratios for this study. Also, we will compare apoE vs LDLR mice on normal and high fat diets.	10 x 3 ratios (normal chow, $apoE^{-/-}$) 10 x 3 ratios (High fat, $apoE^{-/-}$) 10 x 3 ratios (normal chow, $LDLR^{-/-}$) 10 x 3 ratios (High fat, $LDLR^{-/-}$)	10 x 3 ratios (normal chow, $apoE^{-/-}$) 10 x 3 ratios (High fat, $apoE^{-/-}$) 10 x 3 ratios (normal chow, $LDLR^{-/-}$) 10 x 3 ratios (High fat, $LDLR^{-/-}$)	7 days
B. MVE†, Physically Aged	Provide sufficient residence time to transition the particle size from the ~15-20 nm observed near roadway to 100-150 nm observed downwind.	10 total*	10 fresh (30 µg PM/m^3) 10 fresh (100 µg PM/m^3) 10 fresh (300 µg PM/m^3) 10 aged (100 µg PM/m^3) 10 aged (300 µg PM/m^3)	7 days 50 days
C. MVE†, Photochemically Aged	Age emissions in LRRI photochemical reaction chamber.	10 total*	10 fresh (300 µg PM/m^3) (to confirm with previous exposures) 10 aged (100 µg PM/m^3) 10 aged (300 µg PM/m^3)	7 days 50 days
D. MVE† & Urban background	The background atmospheres will include PM (sulfate, nitrate, metals) and gases, including ozone. Details in project 2.	10 total*	10 MVE (300 µg PM/m^3) 10 Background (300 µg PM/m^3) 10 MVE plus background 150 + 150 µg PM/m^3)	7 days 50 days
E. MVE† plus road dust	Resuspended road dust (Phoenix area) with or without MVE gases	10 total*	10 MVE (300 µg PM/m^3) 10 Road Dust (100 µg PM/m^3) 10 Road Dust (300 µg PM/m^3) 10 MVE Road Dust (150 + 150 µg PM/m^3)	7 days 50 days

Note that for these exposures, details regarding development, generation, and characterization will be found in the description of Project 2. †The ratio of diesel:gasolein in MVE for B-E studies will be determined based on the most robust atmosphere from study A. * For studies B-E, we have the capacity to run exposures in parallel, thus reducing the numbers of control groups and also the numbers of exposure periods. Also, the strain and diet will be based on findings from study A.

As Harats et al.[14] found 15-lipoxygenase overexpression to exacerbate altherosclerosis, we wish to investigate whether this enzyme, which oxidizes specific fatty acids in a predictable manner, has a role in observed effects. Thus, similar to objectives in Project 4, we will examine

the generation of 15-HETE and also characterize expression of 15-lipoxygenase (mRNA, protein) in lung, serum, and vascular tissue. Additionally, all pertinent tissues (BALF, serum, erythrocytes and homogenates of lung, aorta and liver) will be saved for lipidomic assessments (described in Aim 2) and pertinent assays related to pulmonary and systemic inflammation.

Statistical Considerations: In general, we propose using 10 mice per group, based on our TBARS data from earlier studies. Based on an $\alpha = 0.05$ and $1-\beta = 0.95$, we are adequately powered to detect a change in TBARS or roughly 5 MDA equivalents. Given that our range of response is generally from around 5-8 MDA equivalents in controls to well over 25 MDA equivalents in MVE exposed mice, this gives us considerable statistical granularity for examining dose response and inter-atmosphere comparisons. As our control animals will be handled identically from study to study, we may be able to pool control results for enhanced statistical power. Typically results will be comparable by one- or two-way ANOVAs, depending on whether concentration-response data are involved. We will work out routine statistical comparisons with GraphPad Prism v 5, and more complicated data sets (i.e., LCMS data) will be analyzed in consultation with the Biostatistics Core.

Anticipated Results: Based on our preliminary data, we predict that the combined motor vehicle exhaust will have a potent effect on lipid peroxidation and vascular pathology, and that while dilution and physical aging will diminish the health effects, photochemical aging will potentiate systemic effects. We hypothesize that the alteration of the phospholipids will contribute to the progression of vascular inflammation due to activation of immune cells via scavenger receptors. The details of these relationships will be addressed in the later Aims. However, in Aim 1, we expect that the presence of ozone, along with numerous other photochemical alterations, will promote the alterations of phospholipids in the pulmonary surfactant, leading to the immune activation and priming of atherosclerotic inflammation.

Specific Aim 2: We will examine effects of the emissions-induced oxidative modifications to endogenous phospholipids (ox-PL), in terms of activating immune-modulating receptors such as LOX-1, CD-36, TLR-2, and TLR-4 and in the formation of innate anti-oxidized phospholipid antibodies. This Aim will utilize receptor deficient models to examine the roles of these receptors, as well as characterize the lipidomic alterations in various tissues.

Rationale: There is a growing appreciation for the role of oxPL in driving the immune responses involved in atherosclerosis. Because we know that 1) near-roadway air pollution is associated with the progression of atherosclerosis in humans and 2) vehicular emissions induce substantial levels of vascular oxPL, we suspect that the modification of endogenous phospholipids may be a central event in air pollution-induced cardiovascular morbidity and mortality. Furthermore, blockade of LOX-1 can abolish the vascular oxidative stress from vehicular emissions exposures (see preliminary data).

To test this, we will:

1. Characterize the patterns of oxidative alterations to endogenous PLs in the surfactant, serum, and aorta, using a lipidomic approach.
2. Measure the amount of innate serum IgG and IgM from exposed mice that bind ox-PLs generated under controlled conditions *in vitro*.
3. Ascertain the role of specific receptors involved in this relationship using receptor-deficient models.

Animal Models: While we will base our final decision on results from Aim 1, we initially propose to use the LDLR$^{-/-}$ model of hyperlipidemia as our working model and background strain, due to the somewhat milder manifestations of hyperlipidemia, and atherogenesis. Alternatively, we may prefer use the apoE model, if the response to emissions exposures in LDLR$^{-/-}$ mice is either too mild or too variable.

We will cross the following receptor deficient strains onto the LDLR$^{-/-}$ background: LOX-1$^{-/-}$, CD36$^{-/-}$, TLR-2$^{-/-}$, and TLR-4$^{-/-}$. The LOX-1$^{-/-}$ and CD36$^{-/-}$ models have both been successfully bred onto the apoE$^{-/-}$ and/or LDLR$^{-/-}$ backgrounds.[12,21,28] Similarly, TLR-2$^{-/-}$ has been crossed onto the LDLR$^{-/-}$ model and MyD88$^{-/-}$ (Myeloid Differentiation protein-88; downstream mediator of TLR-4 transduction) has been placed on the apoE$^{-/-}$ background.[3] We currently have the TLR2$^{-/-}$ and TLR4$^{-/-}$ mice on the apoE$^{-/-}$ background, and it will not be difficult to subsequently switch onto the LDLR$^{-/-}$ background. Note that for all proposed models, the C57BL/6 background strain is consistently used.

Mice will be bred at the University of Washington Animal Research Facility and raised until they are either 12 weeks (if on an apoE$^{-/-}$ background) or 24 weeks (if on an LDLR$^{-/-}$ background). The LDLR$^{-/-}$ mice will also be fed a Western diet starting at 6 weeks of age and continuously thereafter. Mice will be transported to LRRI by a commercial vendor and maintained in quarantine for 14 days prior to exposure. During the quarantine, mice will be simultaneously acclimated to whole-body exposure chambers.

Exposures: For these studies, mice will be exposed to the combined diesel and gasoline mixture (determined in Aim 1), unless a substantial enhancement of vascular TBARS is identified with a different mixing ratio in Aim 1. Exposures will be whole-body and last 6 h/d for 7 and 50 days. Table 3 details the groupings and exposures for this Aim.

Table 3. Groupings and animal numbers for Aim 2

	Filtered Air (Control)	Mixed Vehicle Emissions (100 µg PM/m^3)	Mixed Vehicle Emissions (300 µg PM/m^3)
Wild Type (C57BL/6)	N=10	10	10
LDLR$^{-/-}$ (or ApoE$^{-/-}$)	10	10	10
LDLR$^{-/-}$ x CD36$^{-/-}$	10	-	10
LDLR$^{-/-}$ x LOX-1$^{-/-}$	10	-	10
LDLR$^{-/-}$ x TLR2$^{-/-}$	10	-	10
LDLR$^{-/-}$ x TLR4$^{-/-}$	10	-	10

Preparation of Samples: Surfactant will be isolated from bronchoalveolar lavage fluid in a manner similar to Connor et al.[8] Briefly, following euthanasia and thoracotomy the trachea will be cannulated and the lungs lavaged with 0.15 M NaCl (volume based on body weight, roughly 0.7-1.0 ml) and subsequently two additional times with 80% volume. The pooled lavage fluid will then be centrifuged for 10 min to remove cells (which will be stored to test for activation of macrophages) then again centrifuged at 60,000x*g* for 3 h to isolate the surface-active components. After removing the supernatant, we will resuspend the pellet in 1.0 ml of 0.15 M NaCl, and the lipids extracted as described below. Aliquots of the supernatant will be used to measure changes in the surfactant proteins A-D by immunoblotting (anti-mouse antibodies, R and D systems). Serum will be isolated from whole blood after coagulation by centrifugation for 10 minutes. Aortas will be homogenized and prepared as previously.[25]

For serum, surfactant, and homogenized aortic samples, we will add 1 part chloroform and 2 parts methanol. After shaking, another 1 part chloroform and 1 part water will be added,

followed by centrifugation at low speed for 5-10 min. After removing the lower layer, another 1 part chloroform, will be added, followed by shaking and centrifugation and removal of the lower layer. This step will be again repeated and the combined lower layers will be pooled, washed with 1 M KCl and then with ddH2O. Tubes will be filled with nitrogen and stored at -80c until LCMS analysis.

Endpoints: As with Aim 1, net lipid peroxidation will be assessed with the TBARS assay, to provide an initial and robust indication as to the role of the specific receptor(s) in mediating systemic vascular effects of the inhaled pollutants. In addition, we will also ascertain the changes in specific phospholipids using a liquid chromatography / mass spectroscopy lipidomic approach, as well as with commercially-available kits (15-HETE, others). Briefly, chromatography will be performed either in the normal-phase or in reversed phase. Normal phase chromatography will be performed by injection of phospholipid-containing samples, suspended in chloroform, onto a silica column and eluted with an isocratic mobile phase. UV absorbance will be detected with a diode array detector scanning from 200 to 350 nm. Mass spectrometry (MS) will be performed on an atmospheric pressure ionization (API) triple-quadrupole biomolecular mass analyzer (Applied Biosystems API 4000) equipped with an electrospray ionization source. For internal standards, phospholipids (PAPC and Ox-PAPC) or biological samples are introduced into the electrospray MS by t-infusion or liquid chromatography.

Alveolar macrophages will be obtained from lavage fluid and tested for hydrogen peroxide production before and after stimulation with phorbol 12-myristate 13-acetate using a commercially-available colorimetric kit (R and D Systems), to test the role of surfactant phospholipid activation in these various mouse models.

Lox-1 activation in the macrophages will be determined by measuring activated RhoA/Rac-1. Total RhoA and Rac1 protein and RhoA and Rac1 activity will be measured using kits from Upstate Biotechnology (Lake Placid, NY). Briefly, RhoA and Rac1 activity are measured using rhotekin-RBD and PAK-1 PBD, respectively. These proteins possess binding domains that specifically pull down activated RhoA (Rho-GTP) and Rac1 (Rac1-GTP).[29]

TLR activation will be determined by measuring phospho-IkB-alpha[11] (Cell Signaling Technologies) and CD36 activation will be determined by measuring the phosphorylation of Srk kinase family members Lyn and Fyn and p42/p44 Map kinase[31] (R and D systems). Treatment of the macrophages with ox-LDL (Lox-1 and CD36), LPS (TLR-4) and lipomannan (TLR-2) will serve as the positive controls for receptor activation. Receptor negative macrophages will serve as the negative controls for these assays. Additionally, the aortic sinus will be cryopreserved and sectioned for histopathology of the atheromatous lesion areas. Staining for macrophages and associated immune cells will be conducted, in addition to indicators of endothelial activation (adhesion molecules).

Antibodies that recognize ox-PL will be measured in the serum according to the method of Rolla et al.[37] Briefly, phospholipids will be oxidized with 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH; Polyscience Inc.,Warrington, PA). Cardiolipin, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol will be suspended in phosphate-buffered saline (PBS) pH 7·4 and incubated for 6 h at 37°C in the presence of 1 mmol/l AAPH. These oxidized phospholipids will be extracted with chloroform–methanol (1 : 1 v/v), dried under nitrogen and resuspended at 100 mg/ml in either ethanol or methanol. Antibody binding to oxidized phospholipids will be determined by solid-phase immunoassay. ELISA plates will be coated with the different phospholipid solutions and the solvent will be evaporated.

Non-specific binding sites will be blocked by incubation with 1% (v/v) polyethyleneglycol in PBS pH 7·4. The mouse sera (1:50 dilution in PBS supplemented with 1% PEG) will be added and incubated for 1 h at 37°C. Peroxidase-linked goat anti-mouse IgG or IgM (Dako) will be added and incubated for 60 min at 37°C. Antibody binding will be measured by the addition of a reaction mixture containing 0·4 mg/ml of 1-phenylenediamine, 0·4 ml/ml hydrogen peroxide (30%), 5·1 mg/ml citric acid, 6·1 mg/ml anhydrous Na_2HPO_4, pH 5·0. The reaction will be stopped after 15 min by adding 50 ml 2N H_2SO_4 and absorbance will be measured at 490 nm.
Anticipated Results: The lipid peroxidation assays (TBARS, LCMS, kits) should reveal insight into the net susceptibility to lipid peroxidation, as well as the specific phospholipids that are either created or destroyed by exposure to mixed vehicular emissions. The concentration-response study in wild-type and $LDLR^{-/-}$ mice will engender greater confidence in findings by demonstrating logical trends. Comparing tissues (surfactant versus serum versus aorta) will provide some insight as to whether specific phospholipids are transported or formed in situ, as well as detail the presence of phospholipids that are known to be pro-atherogenic, such as POVPC and POGPC. Increases in anti-ox-PL IgG or IgM will further establish that the vehicular emissions exposure induced oxidation of PLs contributes to systemic immune activation.

By removing specific pattern recognition scavenger receptors, we predict that we will be able to reduce the vascular oxidative burden induced by vehicular emissions in the mouse models of hypercholesterolemia. The relative reduction in aortic TBARS levels will give us an indication as to the prominence of specific receptors in mediating the responses. Additionally, we will be able to delineate the impact of these receptors on vascular inflammation, as indexed by histopathology assays. Ultimately, this Aim will provide a strong link between pollutant-induced modifications of phospholipids, the mediating scavenger receptors, and vascular pathology.

Specific Aim 3: We will further explore the role of immune cell activation in the innate and adaptive responses to emissions-induced phospholipid modifications. In this Aim, we will utilize a mouse model of immunodeficiency, the SCID mouse that lacks mature T and B lymphocytes. Additionally, we will pursue bone-marrow transplants from mice lacking those receptors described in Aim 2 to mechanistically establish the involvement of the multiple receptors that bind oxidatively-modified phospholipids and T and B lymphocytes.

Table 4. Groupings and animal numbers for Specific Aim 3.1

	Filtered Air (Control)	**Mixed Vehicle Emissions**
Wild Type (C57BL/6)	N=10*	10*
$ApoE^{-/-}$ (or $LDLR^{-/-}$)	10*	10*
$ApoE^{-/-}$ x SCID	10	10

Rationale: We have observed that moderate levels of vehicular emissions can promote 1) increased vascular oxidatively-modified phospholipids and 2) infiltration of inflammatory cells into atherosclerotic lesions. *We hypothesize that oxidative modification of certain endogenous phospholipids, presumably in the pulmonary surfactant, can stimulate the activity of immune cells through such receptors and in turn promote the invasion of existing vascular lesions*. In Aim 2 we will establish the role for the specific receptors, but will not delineate whether it is essential to possess the receptors on immune cells or elsewhere.

This study will be divided into 2 sub-Aims. First, we will use the immunodeficient severe combined immunodeficiency (SCID) mouse (Table 4). Second, we will perform bone marrow transplants from wild type, $CD36^{-/-}$, $LOX1^{-/-}$, and $TLR2/4^{-/-}$ mice into the SCID mice to test not only the importance of immune cells but the specific receptors on immune cells (Table 5).

To induce bone marrow aplasia, recipient mice will be exposed to a single dose (950 rad) of total body irradiation 2 hours prior to transplant. Bone marrow-derived cells will be isolated from the donor mice by flushing the femurs and tibias with phosphate-buffered saline. Cell suspensions will be made by thoroughly passing cells through a 5 ml sterile glass pipette. The irradiated recipients will receive 1 X 10^7 whole bone marrow cells in a total volume of 400 µl PBS by intravenous injection into the tail vein.

Endpoints: Similar to Aim 2, we will examine changes in lipid peroxidation, both in terms of oxidatively (TBARS, LCMS) and enzymatically (15-HETE) modified phospholipids. We will assess lipidomic changes in surfactant, serum, and aorta. Additionally, we will assess vascular lesion histopathology, again focused on the presence of macrophages, lipids (oil red O) and lipid byproducts (4HNE). With the cellular compartment of the BALF, we will again test the activation of alveolar macrophages with the hydrogen peroxide and phosphorylation assays.

We will need to assess circulating monocyte populations following bone marrow transplantation to ensure adequacy of the model. Because we will have the flow cytometry assays prepared for these studies, we will also compare the influence of the mixed emissions on immune cell populations (T cells, monocytes, etc). This will not only be informative for the present study, but may provide important data for future studies on chronic effects of roadway-derived air pollutants.

Table 5. Groupings and animal numbers for Specific Aim 3.2

	Filtered Air	Mixed Vehicle Emissions
ApoE$^{-/-}$ x SCID	N=10	10
ApoE$^{-/-}$ x SCID + BM^{WT}	10	10
ApoE$^{-/-}$ x SCID + BM$^{CD36-/-}$	10	10
ApoE$^{-/-}$ x SCID + BM$^{LOX1-/-}$	10	10
ApoE$^{-/-}$ x SCID + BM$^{TLR2/4-/-}$	10	10

Anticipated Results: Naturally, we expect that the magnitude of vascular lipid peroxidation and inflammation will depend largely on the scavenger receptor(s) that most prominently convey the inflammatory signal. SCIDxApoE$^{-/-}$ mice should display a marked diminution of lipid peroxidation and obviously reduced vascular inflammation. By returning bone marrow and reconstituting the immune system in these mice, we should recover the systemic vascular effects of the air pollutants. However, by using bone marrow from animals lacking CD36, LOX-1, or TLR2/4, we should observe variations between the immunodeficient and immune-reconstituted mice that indicates the relative importance of these receptors on T and B lymphocytes and leukocytes in driving said vascular toxicity.

EXPECTED RESULTS, BENEFITS, OUTPUTS, AND OUTCOMES

This project will generate innovative findings regarding the role of fresh versus aged vehicular emissions on vascular health. The generation and examination of the impact of aging on fresh, mixed vehicular emissions has never been conducted previously, is not easily conducted anywhere else, and is vital to our understanding of the near roadway health effects phenomena observed in population studies. In addition to investigating entirely novel mixtures, we will also detail the role of the immune system, both in terms of receptor and cellular subtypes, in mediating the systemic vascular impact of air pollution exposure. The output of this project will include both publications reporting the findings, as well as providing methods for generating innovative complex atmospheres for toxicological inquiries. Lastly, we feel that this project, in concert with Projects 2 and 4, represents the next step in integrative research, as we will develop a translational research model that is iterative and multidisciplinary that further builds on our ongoing relationship between the involved institutes and researchers.

GENERAL PROJECT INFORMATION

This proposal will necessitate the expertise of the three institutes involved. Study direction, design and interpretation will be shared between Matthew Campen at UNM and Michael Rosenfeld at UW. Exposures, along with some biological assays, will be conducted at the LRRI. Breeding and bone marrow transplant experiments will be conducted at UW, along with analysis of phospholipid alterations and histopathology.

Matthew J. Campen, Ph.D., M.S.P.H. (co-principal investigator, 15% effort) is an experienced investigator in the cardiovascular health effects of air pollution. He has been studying the health impact of several complex combustion atmospheres for the past 7 years.

Michael E. Rosenfeld, Ph.D. (co-principal investigator, 5% effort) is an internationally-recognized expert in vascular pathology, with an established interest in applications related to air pollution. His background in the basic underpinnings of atherosclerosis promotion and plaque remodeling will be essential to the development and conduct of the present study.

Amie K. Lund, Ph.D. (co-investigator, 10% effort for years 1-3) currently runs the Cardiopulmonary Physiology Program at LRRI and is instrumental in managing exposure studies for basic and applied research projects.

Jacob D. McDonald, Ph.D. (co-investigator, budgeted in Project 2) manages the Aerosol Chemistry program at LRRI and is the principal investigator of Project 2, which is the sister project of Project 3. In addition to generating exposures atmospheres, he will provide resources and expertise for bioanalytical lipidomic assays.

Schedule and Timeline: Coincident with Project 2, we will conduct the bulk of exposures in years 1-3, especially those related to the photochemical and physical aging of the vehicular atmospheres. Latter years will be used for more detailed assays and follow-up exposures, potentially exploring higher or lower concentrations, or conducting any of those contingency exposures described in Aim 1. The studies described in Aims 2 and 3 will also be coincident with Aim 1, as during the genesis of more complex transformed atmospheres, we will always be able to siphon the fresh, mixed diesel and gasoline emissions for a consistent parallel exposure.

Study Management: Dr. Lund will organize the procurement of animals from the University of Washington, schedule exposures and necropsies, and disseminate collected tissues to the appropriate laboratory. Dr. Lund currently manages a number of studies in this capacity, including the NPACT study, which also requires integration with UW. Drs. Campen and Rosenfeld will be responsible for the design, data compilation, analysis, and communication of research findings. Data will be stored digitally in parallel between all three institutes to ensure adequate sharing of findings and redundancy in storage. Periodic conference calls will be held to communicate proceedings and findings, based largely on the organization/scheduling of exposures and all investigators will meet annually in person.

Facilities: The combined facilities are superb for the execution of the project. Details regarding the exposure facilities can be found in Project 2. In brief, the Chronic Exposure Laboratory has consolidated resources for a multitude of inhalation exposure atmospheres, from single gases (e.g., carbon monoxide) to complex combustion emissions (e.g., diesel) to transformed atmospheres (photoreaction chamber). Additionally, we can combine atmospheres, filter PM from complex emissions, or denude reactive gases. The animal facilities at LRRI, UW, and UNM are AAALAC-approved, and all studies will be conducted with approval from the germane Institutional Animal Care and Use Committees. The Bioanalytical Chemistry Core at LRRI and the UW Center for Ecogenetics and Environmental Health Proteomics and Metabalomics Core both have the expertise and numerous spectrophotometric systems for running required analysis of phospholipids.

Project 4: Vascular Response to Traffic-Derived Inhalation Exposure in Humans

1. Objectives

Air pollution exposures are associated with ischemic heart disease. Recent observations, including from our research group, demonstrate that traffic-related air pollutants acutely trigger increased arterial reactivity, vasoconstriction, and increased blood pressure in human and animal models. These observations can be exploited to examine both acute and chronic health effects of air pollutants that are due to activation of a cascade of biological pathways still to be elucidated.

In this project, we will use our established inhalational exposure facility and pertinent experience in human exposure studies to advance the Center's research agenda with a double-blind, controlled exposure crossover clinical study in 24 subjects, randomized to order. Using an innovative approach in which contrasts in exposure, use of pharmacologic blocking agent, and participant susceptibility by genotype are nested in the experiment, we can address several hypotheses in this study. Building on our prior work we will use controlled clinical exposures to confirm or determine whether:

- traffic (e.g., diesel and gasoline engine exhaust) -derived aerosols exert demonstrable and important acute vascular effects in human subjects;
- traffic-derived aerosols acutely induce increased lipid peroxidation, response to oxidized phospholipids, and result in measurable impacts on gene expression and DNA methylation, *in pathways that are related not only to the triggering of acute cardiovascular events, but also to the development and progression of atherosclerosis*; and
- these *biological effects will be greater due to mixtures of secondary aerosols* identified as more accurately reflecting real-world traffic-related exposures and as having greater biological potency through work in Center Projects 1, 2, and 3.

These studies will be largely launched in Center Year 3, guided by findings generated in Center Projects 1-3, with exposure atmosphere generation led by the Project 2 team. As a result there will be the opportunity to modify the choice of exposure, population to be studied, specific outcomes, and pharmacologic agents to more accurately reflect the state of the science in 2012 when human exposures will begin.

Hypothesis 1 (Specific Aim 1): Acute exposure of human subjects to combustion-derived pollutants will result in brachial artery vasoconstriction, increased circulating endothelin-1, and increased systolic blood pressure, with toxicity predicted by other center projects.

Specific Aim 1a: Inhalation of combustion-derived pollutants of higher potency, as identified through projects 2 and 3, will result in enhanced brachial artery vasoconstriction, increased circulating endothelin-1, and increased systemic blood pressure, compared to an equivalent concentration of combustion-derived pollutants with contrasting characteristics or to filtered air.

Specific Aim 1b: Pollutant-related changes in blood pressure and vasoconstriction will be increased in subjects with an established genetic polymorphism in the lipoxygenase ALOX15 (G allele at SNP G189C, rs2664593).

Hypothesis 2 (Specific Aim 2): Acute exposure of human subjects to traffic-related combustion- derived pollutants will result in evidence of lipid peroxidation and pro-atherogenic gene transcription and epigenetic changes, with toxicity predicted by other center projects.

Specific Aim 2a: Inhalation of air pollutants of higher potency, as identified through projects 2 and 3, will result in increased plasma oxidized LDL, malondialdehyde, and anti-phospholipid antibodies, compared to an equivalent concentration of combustion-derived pollutants with contrasting characteristics or to filtered air.

Specific Aim 2b: Inhalation of air pollutants of higher potency will result in measurable early changes in monocyte gene transcription, as evidenced by modified mRNA expression of genes associated with oxidative stress (e.g., heme oxygenase [HMOX1] and the glutamate cysteine ligase catalytic subunit [GCLC]) and lipid metabolism (e.g., peroxisome proliferator-activated receptor α [PPARA]) in circulating CD14 positive cells, compared to filtered air.

Specific Aim 2c: Pollutant-related changes in gene transcription will be attenuated or eliminated by use of a pharmacologic blocker of lipid peroxidation, alpha-lipoic acid.

Specific Aim 2d: Inhalation of traffic-derived pollutants will result in measurable changes in DNA methylation status in circulating lymphocytes, including an increase in hypermethylation of FOXP3, a key gene in differentiation of regulatory T cells.

2. Approach / Activities

Background and Preliminary Data

Air Pollutants, Especially Traffic-Related Air Pollutants, Cause Increased Vascular Reactivity, Vasoconstriction, and Increased Blood Pressure

Air pollutants have been associated with changes in vascular tone and endothelial function, suggesting a pathway by which air pollution can induce adverse cardiovascular outcomes such as myocardial infarctions. For example, vasoconstriction has been documented in healthy human subjects following controlled exposure to concentrated ambient particles.[1] In another study, non-experimental exposures to ambient particles were linked to changes in vascular reactivity and endothelial function in diabetic subjects.[2] Similar results were found specifically with diesel exhaust (DE) at 300 µg/m^3, with controlled exposures inducing changes in vasomotor responses in healthy human volunteers.[3] These findings have been supported by the toxicology literature with pollution exposures being associated with increased vasoconstriction responses demonstrated in rats[4, 5] as well as enhanced levels of endothelin.[6, 7]

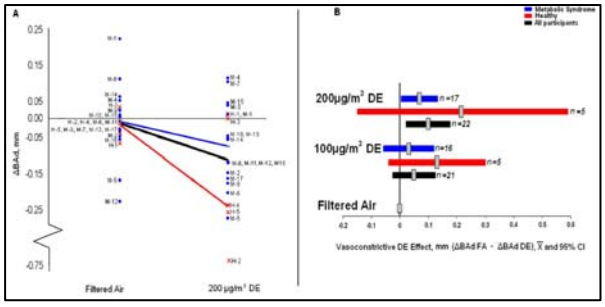
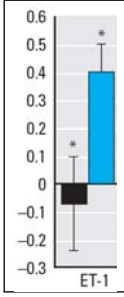


Figure 1: Vasoconstriction with Diesel Exhaust Inhalation
A. Changes in brachial artery diameter (BAd) following exposures to 200µg/m^3 DE or FA; lines represent mean Δ BAd (pre-to-post) at each exposure level. B. Dose-response relationship of diesel exhaust effect on brachial artery diameter. Bars show mean and 95% confidence interval for vasoconstrictive effect for two study sub-populations and overall group. Wide confidence intervals for healthy group reflect small sample size and not higher variance.

Our group has completed a set of experiments with human subjects exposed to DE at 100 and 200 µg/m^3 for two hours, and found conductance artery vasoconstriction with a dose-related effect, with an average 0.11 mm greater degree of constriction of the brachial artery following 200 µg/m^3 (DE_{200}) than exposure to filtered air ($p=0.01$).[8]

We also found increased levels of plasma endothelin-1, an endogenous vasoconstrictor 100-fold more potent that norepinephrine, which is produced by the vascular endothelium in response to stress, as shown in Figure 2.[8] The endothelin system plays a major role in regulation of vascular tone and blood pressure.[9, 10]

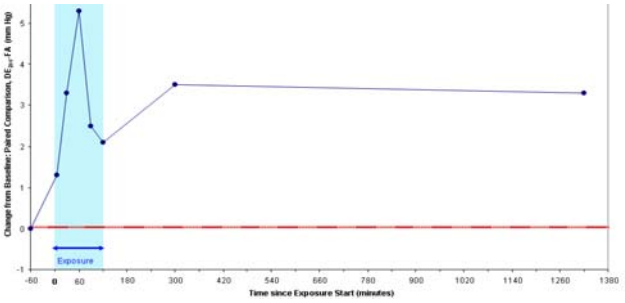


Biomarker	FA		200 μg/m³ DE	
	Preexposure	Postexposure	Preexposure	Postexposure
ET-1				
Healthy (n = 6)	1.47 ± 0.21	1.35 ± 0.27	0.86 ± 0.06	1.62 ± 0.18
Metabolic syndrome (n = 16)	1.35 ± 0.17	1.41 ± 0.15	1.31 ± 0.18	1.62 ± 0.18
All participants (n = 22)	1.38 ± 0.13	1.39 ± 0.13	1.19 ± 0.14	1.62 ± 0.13

Figure 2. Graphic: Fold-change in log-transformed plasma endothelin-1 levels from pre-exposure to 3-hrs after initiating exposure to either filtered air (black) or DE_{200} (blue). p=0.01. Table: Mean values, in pg/ml.

More recently, by analyzing our records on the 48 subjects exposed to DE_{200} and filtered air (FA) in crossover experiments in our controlled exposure laboratory thus far, we have found a significant rapid effect of DE_{200} on systolic blood pressure (SBP), to a greater extent than diastolic blood pressure. In comparison to measurements taken each session before the exposure session, the mean difference between SBP DE_{200} and FA is positive beginning shortly after the session begins and throughout our 24 hours of follow-up, and reaches its peak in magnitude (5.3 mmHg, 95% CI 1.5,9.2; p=0.008) following one hour of exposure. This represents a clinically significant effect on an outcome of unambiguous importance.

Figure 3. Mean diesel effect on systolic blood pressure (n=49; all available subjects; all 4 protocols)



Regulation of Vascular Function and Endothelial Function

The understanding of mechanisms underpinning vascular responses is a rapidly advancing field. The integral role of inflammation, endothelial function, and lipid peroxidation in both acute and chronic cardiovascular disease processes is under intensive study. It is now abundantly clear that atherosclerosis is an inflammatory state, in which the endothelium and leukocytes are actively involved. Further, it is increasingly clear that these processes which control endothelial function on a moment-to-moment basis also are integrally involved in the progression (extent) and activity (plaque vulnerability) of atherosclerosis. Hence, further investigation of these interactions, and the role of environmental exposures like traffic-related air pollutants in perturbing these delicately balanced interactions, is critically important to understanding chronic as well as acute sequelae.

Harnessing Information from Genomic and Epigenomic Variation to Understand Underlying Mechanisms

Atherosclerosis, ischemic heart disease, and triggering of myocardial infarction are a complex set of disease process with numerous phenotypes and certainly many genetic loci. Genetic variation in humans and animals can be exploited to unravel specific questions regarding mechanisms and susceptibility. The use of transgenic strains has accelerated this process for murine research. Increasingly sophisticated genotyping and bioinformatic strategies and completion of mapping of the human genome are making human research along these lines more fruitful as well. The use of a specific environmental agent (DE) in a controlled exposure permits a physiological perturbation with a sufficiently narrow spectrum that we can learn directly from the genetic variation what pathways are involved. We believe that this approach may lead to understanding not only of the environmental disease process, but more basic biological processes with broader

implications. While investigations of these effects with regard to air pollution health effects are still largely in an exploratory phase, we are able to address these issues in an integrated hypothesis-driven manner based on preliminary results from our studies to date.

Genomic Variation Demonstrated through Examination of Gene x Environment Interactions in Cardiovascular Effects of Near-Roadway Exposures

We have demonstrated a significant independent impact of residence near a roadway (< 50m) on increased left ventricular mass (LVM), a sign of important end-organ damage (often related to hypertension) predictive of development of both ischemic heart disease and congestive heart failure, in the Multi-Ethnic Study of Atherosclerosis.[11] More recently, our group examined tagged SNPs in 12 selected candidate genes with common variants to determine if they modify the effect of roadway exposure on LVM, adjusting for multiple potential confounders and accounting for multiple testing (submitted for publication).[12] We found significant SNP-traffic exposure interactions on left ventricular mass for variants in lipoxygenase-15 (ALOX15) and the type-1 angiotensin II receptor (AGTR1); lipoxygenase-15 provides the most insight into the pathways being explored in Project 3, so will be a focus in this proposal. In particular SNP rs2664593 (G-189C) in the 5' promoter region of the lipoxygenase-15 gene, strongly modified the effect of roadway proximity on left ventricular mass. Living within 50m of a major roadway and carrying the CC (minor allele homozygote) at this locus was associated with an 8.5 % difference (95% CI 2.6, 14.9) in left ventricular mass in the fully adjusted model, compared to a -1.7 % difference for those with GG (*p* for interaction 0.003). For comparison, an increase in systolic blood pressure of 5 mmHg was associated with 1.6% increase in left ventricular mass in this population.

Lipoxygenase-15 is an oxidizing enzyme that can produce reactive lipid hydroperoxides during arachidonic acid metabolism, and has a complex role in atherogenesis—appearing to play a role in early atherosclerosis but not late in the process.[13, 14] Variants at this locus have previously (but inconsistently) been associated with carotid atherosclerosis and to interact in the relationship between carotid wall thickness and insulin resistance.[15, 16] We believe it is a highly promising variant to use in selection of participants for our controlled exposure studies.

Early gene expression effects of traffic-related exposures

Environmental exposure-related changes in gene transcription is a promising way to determine specific pathways involved in health effects. We have piloted the use of genome-wide expression arrays, using peripheral blood mononuclear cells in our human subjects, comparing changes in expression pre-exposure to selected times post exposure DE_{200} vs. filtered air in five subjects.[17] We found several provocative, though preliminary, findings indicating selective DE-related differences in genes in pathways of interest supported by other toxicological results. Three findings can be highlighted and will be specifically followed up in the proposed studies.

Glutamate cysteine ligase catalytic subunit (GCLC) is a component of the rate-limiting step of glutathione synthesis, regulated by feedback inhibition exerted by glutathione on GCL and by the glutathione redox system. Effective regulation of glutathione activity involves several enzymes directly involved in its synthesis and cycling, as well as other enzymes in associated pathways of intermediary metabolism. We observed a 1.8 fold decrease in expression of GCLC at 6 hours post-exposure initiation and 1.5 fold decrease the morning following exposure to DE2000

compared to filtered air. If confirmed, this indicates a disruption of the response to oxidative injury, as has been observed with some toxic exposures such as TGFβ1 induced apoptosis.[18]

Heme-oxygenase-1 (HMOX1) is an antioxidant enzyme that catabolizes heme to produce carbon monoxide and biliverdin. It has been previously observed that diesel exhaust particles, as well as oxidized phopholipids, induce HMOX1 expression in macrophages *in vitro*.[19] We observed a 2.0 fold increase in expression of HMOX1 in PBMCs the morning following exposure to DE200 compared to filtered air.

Peroxisome proliferator–activated receptor alpha (PPARA) is a member of the PPAR nuclear receptor family, appears to play a central co-ordinated role in the regulation of fatty acid oxidation, lipid and lipoprotein metabolism and inflammatory and vascular responses.[20] In addition to the well-known effects on lipid metabolism, diabetes, and obesity, they also seem to affect ET-1 signaling in endothelial cells and vascular remodeling in smooth muscle cells.[21] Somewhat surprisingly, since increased PPAR-α would be considered anti-atherogenic, we observed a 3.5 fold increase in expression of PPARA the morning following exposure to DE_{200} compared to filtered air. This observation requires confirmation and further examination. Importantly lipid peroxidation, as through oxidized LDL, implicate PPAR-α in the maintenance of monocytes at sites of inflammation such as the atherosclerotic lesion.[22]

We propose to focus on expression results for these genes, selectively in monocytes, in this proposal. Circulating monocytes play a key role in inflammation,[23, 24] and represent one of the most relevant (and easily accessible cell types) to target for expression investigation into atherosclerosis.[22] These cells bind to activated endothelium and migrate into the subendothelial space as one of the initiating events in atherogenesis. After transformation to macrophages, they produce chemokines and cytokines which promote atherosclerotic plaque formation, and proteases which may lead to plaque rupture and clinical events such as heart attack.[25, 26] Macrophage-derived foam cells in the atherosclerotic lesions have expression phenotypes that are similar to those of circulating monocytes, but not of resident adventitial macrophages.[27] Thus, it is very plausible to consider that expression changes in circulating monocytes could influence (or at least reflect) the pathogenesis of atherosclerosis. Further, this is consonant with the Project 3 hypothesis which anticipates monocytic invasion of existing vascular lesions as the eventual result of traffic pollutant exposures.

Increased Lymphocyte DNA Methylation at FOXP3 provides insight into environmental influences on atherosclerosis

It is increasingly well understood that epigenetic changes including DNA methylation can regulate diverse cellular functions, including the regulation of inflammatory gene expression, and cell proliferation processes important in atherosclerosis. It has been demonstrated (in animal models, at least) that certain environmental exposures can interact with specific DNA sequences to produce persistent epigenetic modifications that can influence gene expression and presumably susceptibility to disease.[28] Most attention to epigenetic research has been focused on early life exposure until some recent studies from Baccarelli and colleagues.[29, 30] They have demonstrated changes in "global" DNA methylation (long interspersed nucleotide element (LINE)-1 and Alu repetitive elements) with relatively small daily-scale exposures to ambient traffic-related air pollutants and decreased iNOS promoter DNA methylation with (larger) particulate exposure excursions in steelworkers. Data developed in our own laboratory) has also demonstrated the potential of using this approach to understand these health effects. As part of an

ongoing study, DNA methylation assays were performed for 5 subjects before and after controlled exposure to both diesel exhaust and filtered air exposures, on PBMCs for a small panel of candidate genes with highly variable degrees of methylation (Forkhead Box P3 [FOXP3], neutrophil elastase [ELA2], and Interferon regulatory factor 1 [IRF1]). We found that, in the FOXP3 gene, the proportion of regions demonstrating hypermethylation was elevated after diesel exhaust treatment, relative to after the fresh-air control (mean difference in morning-after-exposure vs. before-exposure percent hypermethylated: 7.1%, p = 0.03; all subject-level pairwise differences were positive.) Recent evidence implicates CpG methylation for T regulatory cell development and function, especially through Foxp3.[31]

Blockade of lipid peroxidation provides insight into mechanisms involved

Alpha-lipoic acid is a readily available dietary supplement with strong antioxidant capacity, with USP grade material available, which has been evaluated (and is in wide use internationally) for several indications including treatment and prevention of diabetic neuropathy.[32]. It also improves endothelial function in the Metabolic Syndrome.[33] It is also well-tolerated, with an excellent safety profile in doses found to be effective for these indications (such as 600 mg orally per day). Using a pharmacologic blocker of lipid peroxidation can help determine whether downstream effects of pollutants are mediated by their oxidant properties.

Research Methods

We will use a well-characterized human exposure facility, customized to reflect findings in Center Projects 1-3, to examine effects of diluted combustion-derived exhaust, in a double-blind, controlled crossover experiment, randomized to order.

Building on data derived from animal studies and exposure characterization studies (Projects 1-3) in Center years 1 and 2, and by customizing exposures to capitalize on those findings, we propose clinical experiments to be primarily conducted in Center years 3 and 4. In healthy subjects, we will test whether a traffic-derived laboratory-generated pollution atmosphere, as suggested through other Center projects, causes an increased vascular response (brachial artery vasoconstriction and increased blood pressure) and changes in several parameters collected from peripheral blood, compared with both a roadway-derived pollutant mix of apparently lower potency, and with filtered air.

While we plan to adjust our approach to reflect the results of the animal experiments and interval scientific knowledge, we now propose to determine if specific downstream effects are blocked by the antioxidant alpha-lipoic acid, and to determine if traffic-related pollutants' vasoconstrictive effects are increased in subjects with a common SNP variant in the gene coding for the lipoxygenase ALOX15, a variant which is associated with development of early atherosclerosis.

Timeline. Project 4 will conduct pilot studies and procedures to customize the exposure conditions in Center years 1 and 2, and the main experimental procedures will be conducted in Center years 3 and 4. During Center year 5 we will complete sample and data analysis and the production of manuscripts.

Key Design Elements for Human Experiments.

Each experiment involves controlled exposures to healthy adult subjects aged 18-49, is crossover in design, and is randomized and counter-balanced in order of exposures and treatments. In the

crossover design, **each subject receives each experimental condition and treatment, with sufficient washout between exposures and treatments, allowing paired analysis and creating an extremely efficient and statistically powerful study**. Since this experiment includes selection for genetic susceptibility, randomization to order of exposure/treatment occurs within each genotypic group. Exposure sessions are 120 minutes in duration. Each subject's experimental sessions will occur at the same time of day and there will be at least 14 days between sessions. The participant, all staff with participant contact, and investigators assessing outcomes will be blinded to exposure situation and pharmacologic agent. For the pharmacologic interventions involved, we follow standard clinical practice for blinding of all personnel and staff, until the experiment is completed.

The exposure system is operating and at steady state prior to subject arrival. Subjects are fasting for at least 8 hours before arriving for their experimental session. Each subject has all procedures performed at the same time for each of their sessions.

The experiment uses a five-condition design (HP + placebo, HP + lipoic acid, LP + placebo, FA + placebo, FA + lipoic acid). This experiment will include 24 subjects, with balanced genotype (8 with CC, 8 with GC, 8 with GC at the ALOX15 SNP G-189C) polymorphism, and two-hour exposures to filtered air or DE. Subjects will take oral alpha lipoic acid, or matched placebo, prior to a session (dosing scheme below).

Subject Selection and Recruitment

All subjects must be nonsmokers with no history of hypertension, asthma, diabetes, hypercholesterolemia, or other chronic condition requiring ongoing medical care. Subjects are recruited from advertisements in the university and community. Subjects are paid a per-session reimbursement for their time, with an additional deferred reimbursement for completing all exposure sessions.

Subject Screening. Subjects who meet the eligibility criteria above will be invited for a screening visit, which will be conducted after an overnight fast. At this visit, the experiment will be explained, and information provided in order to obtain informed consent to participate. Following informed consent, a screening interview including medical and medication history is conducted. Each prospective subject will undergo measurement of height, weight, fasting blood sugar, blood collection for genotyping and lipid profile, resting blood pressure, and 12-lead electrocardiogram. If the subject meets screening criteria based on these measurements, they will be scheduled for the ultrasonographic assessment of brachial artery reactivity protocol, described below. Subjects who have body mass index greater than 25, elevated fasting blood sugar, bradycardia, hypotension, evidence of a medical condition requiring treatment, or who do not have a normal or clearly measured FMD response, will be excluded from further study.

Women of childbearing age will undergo a serum pregnancy test at screening, and will be instructed to practice effective contraception during the study. We require a negative pregnancy test prior to participating in exposure sessions or taking study medications.

Selection for Genetic Susceptibility Factor.

Results of the prior animal and human studies will inform selection of the appropriate susceptibility genotype. As our goal for this aim is to further dissect the mechanism of cardiovascular effects of traffic-related pollutants in humans, our genotype will reflect a more specific pathway of effect, At this time, we plan to select subjects with the ALOX15 variant as

discussed previously, but reserve the option to choose an alternate SNP. This is a rapidly changing area, and genetic variants felt to be important today may well be considered unimportant in two years as finer mapping of ALOX15 becomes available; further, it is likely that continued advances will have identified important haplotypes (with tagging SNPs) for important phenomena, and we will want to adjust our plans accordingly. In order to address logistics of recruiting, we prefer a SNP with a minor allele frequency that is at least 0.2 or a haplotype that is present in at least 20% of the population, though biological systems are not always accommodating in this regard.

8 of the 24 subjects will be recruited to have each of the CC, GC and GG genotype for the gene ALOX15 SNP G-189C (rs2664593). Subjects are tested using DNA extracted from whole blood, using methods well-established in the Functional Genomics Laboratory,[34] which has performed thousands of genotyping studies in support of human and animal environmental research. Since we anticipate that as few as 5 per cent of our subject pool will have the GG genotype at this locus, we anticipate needing to screen substantially more subjects (nearly 200 in all) than will be studied in the experiment. We have had good success with this approach in a current study with a similarly frequent minor allele.

TaqMan SNP genotyping method. SNP genotyping will be carried out using 5'-nuclease assays which employ specific fluorogenic TaqManTM probes (PE Applied Biosystems, Foster City, CA). A region of approximately 200bp in length surrounding the SNP site will be imported into PrimerExpressTM software (PE Applied Biosystems). Specific PCR primers will be designed using this software and purchased from MWG Biotech (High Point, NC). All probes will be 3'-labeled with the TAMRA quencher dye. In addition, the specific wildtype and variant probes will be 5'-labeled with the 6-FAM reporter dye and the VIC reporter dye, respectively, and will be purchased from Integrated DNA Technologies (Coralville, IA). The fluorescent 5'-nuclease assays will be performed and analyzed on an ABI PRISMTM 7700 Sequence Detection System (PE Applied Biosystems). The specific PCR reaction conditions will be based on the general guidelines provided by the manufacturer and incorporated 35-75ng of genomic DNA template.

Exposure Methods. We have demonstrated that our UW laboratory system can effectively create a diesel exhaust particulate exposure for inhalation that mimics, to the extent feasible, realistic ambient characteristics of exposures from contemporary heavy-duty on-the-road diesel engines using currently available fuel, and serve as an effective model for evaluating health effects of pollutants. Exhaust dilution engineering is in place and we have confirmed that we can create stable and well-characterized exposures to DE.[35] We have used this system for more than 150 human exposures to date with excellent results and no adverse events.

This project will customize the facility to create two new exposure atmospheres, both of traffic- and roadway-derived aerosols, with one of "high potency" and one of "low potency". Several aspects of customization, including alteration to combustion devices, and aging of exhaust stream, have been pilot tested. In this project, this customization will be directed by Center Project 2 investigators to achieve and characterize atmospheres as described in that Project.

In this project, we use the terms "high potency" (HP) and "low potency" (LP), though this represents an oversimplification of the differences in the environments. We will apply these two environments in a comparable manner, such that the overall concentrations of pollutants ($\mu g/m^3$ of $PM_{2.5}$) are the same in both the HP and LP environments. These environments will be used in contrast to our filtered air (FA) environment, as described below.

Blinding. While somewhat surprising, we (like other investigators such as the Umeå group) have determined that subjects are unable to accurately identify the concentration of diesel exhaust in the exposure facility; our subjects do only a bit better than chance in identifying the exposure (filtered air or diesel exhaust) that they received. As a result, though blinding is imperfect, we are able to maintain essentially double-blind procedures.

Exposure Facility

Human exposures occur within an existing, well-characterized 100 cubic meter exposure chamber. Previous measurement and tracer studies indicate that the chamber can produce approximately ideal plug flow under suitable inlet temperature conditions.[36] All human subject exposures and exposure characterization occur in a 13.8 cubic meter central breathing and sampling zone. The air entering the room is conditioned to 18° C and 60% relative humidity. To maintain steady exposure concentrations and safety for human subjects, during each session we collect ongoing measurements using continuous reading instruments (TEOM, PSAP, 2 CPCs, CO monitor (Lear Seigler 9300), and NO_x monitor (API 200A chemiluminescent). Feedback from continuous nephelometry also provides ongoing adjustment of the dilution system.

Experimental Procedures

Exposure Session Protocol

The course of a session is shown in Table 1. Female subjects' sessions are timed with regard to menstrual cycle. Following an overnight fast, the subject will arrive at the Clinical Research Center for placement of an intravenous catheter for venous access in the non-dominant arm, initial blood draw and placement of saline lock. Blood pressure monitoring with a portable, recording noninvasive blood pressure monitor is also initiated (opposite arm from brachial artery measurements) and is in place until the following morning. The subject then undergoes brachial artery studies and then is transferred to the exposure facility.

Table 1. Exposure Session Timeline for Human Studies

Study Element	-8	-2	-1	0	1	2	3	4	5	6	14	24
Begin 8-hour fast	X										x	
Blood collected			X		x		X		x		x	X
Exposure												
Brachial artery testing												
Blood Pressure Monitoring												
Defined composition meal							x					

Hours (from start of exposure session)

Hours –7 to –3, 7 to 13, and 15 to 22 not shown. Large X are timepoints for DNA/RNA isolation

On arrival in the laboratory, subjects will be started on continuous pulse-oximetry, electrocardiographic, and respiratory rate monitoring, and along with blood pressure these parameters are monitored by the technician throughout the exposure.

The subject is seated comfortably, semi-recumbent, in the chamber throughout the two-hour exposure session. Blood is drawn from the venous catheter for analysis 1.5 hours into the exposure.

Following the exposure protocol, the subject is transported for brachial artery diameter assessment, and then to the General Clinical Research Center (CRC) for continued observation. Except for water during the exposure session, subjects fast until completing the ultrasound assessment, when they eat a defined composition meal. Identical meals are provided at each visit (matched to actual consumption at first session). Blood is collected at 4 hours post-exposure (6 hours after the exposure began), then the catheter is removed, and the subject discharged. The

subject returns the following morning for collection of fasting blood and first morning voided urine specimens.

Pharmacological Intervention

We will administer the anti-oxidant lipoic acid to participants. Each subject will take 600 mg of lipoic acid (or matched placebo) 24 hours prior to the exposure session and an additional dose of 600 mg of lipoic acid 1 hour prior to initiating exposure. Alpha lipoic acid, USP, will be procured via the Investigational Drug Service (IDS) at the University of Washington Medical Center, which will participate in the randomization of subjects to treatment regimens, and prepare the active capsules and matched placebos using pharmaceutical grade materials. Previous studies demonstrating vascular (including brachial artery) and microvascular (diabetic) effects in human subjects have used between 300 and 600 mg per day with an excellent safety profile.[37]

Assessment of the Brachial Artery

Ultrasonographic assessment of the brachial artery, including assessment of flow-mediated dilation (FMD), provides *in vivo* assessment of endothelial and smooth muscle activity, and has become an established experimental method. A strict research protocol is required; guidelines have been published,[38] and we enhance these guidelines by implementing detailed standard operating procedures and computerized image analysis based on our own work.[39] We use an ATL 5000 ultrasound instrument (Philips/ATL, Bothell, WA) and a high frequency linear transducer with a range from 7-12 MHz. The technician for this study has 15 years experience as a research sonographer; over the last ten years she has conducted thousands of brachial artery FMD studies per year in research protocols.

The subject rests supine for 5 minutes before starting the procedure. Baseline brachial artery diameter measurement is made 3 cm proximal to the antecubital fossa. The position of the transducer on the arm is marked using adhesive markers. (*Brachial artery diameter*, and the change from pre-exposure value, is the main outcome measure for the vasoconstriction measure.) A blood pressure cuff on the forearm is inflated to a pressure 50 mmHg above the baseline systolic pressure and is held constant for 5 minutes. The cuff is then deflated and removed from the subject's arm, which results in reactive hyperemia, shear stress and brachial artery dilation. The maximal dilation measured in the following 2 minutes, compared to baseline, is reported in millimeters and as a percent changed from the baseline measurement, to determine endothelium-dependent FMD.[40] Following 20 minutes of rest, the subject is given 0.4 mg of sublingual nitroglycerin, as an exogenous NO donor. Peak vasodilation in the subsequent 3-minute interval determines endothelium-independent vasodilation. We use ECG gating of ultrasound images, with a MatLab interface for managing data acquisition. Image analysis is completed by one staff member, blinded to exposure status, using Brachial Tools software (Medical Imaging Applications, Coralville, Iowa).

Blood collection and preparation

Blood samples are drawn from the established 19-gauge intravenous catheter into appropriate tubes at each time as shown in Table 1, and prepared according to assay requirements. Subjects are always at rest in the semi-recumbent position for at least 30 minutes prior to blood draw. Samples will be coded for blinded analysis. Serum, EDTA plasma, and citrated plasma are

obtained by centrifugation of the whole blood collection mixtures at 4°C (1,800 x g) for 15 minutes. Aliquots of serum and plasma are stored in cryotubes at -70° C for batch running.

Nuclear material will be extracted from the samples collected prior to exposure, 3 hours after initiation of exposure, and the following morning (24 hours after the initial sample is drawn). We have successfully collaborated with the department's Functional Genomics Laboratory (Fred Farin, director) for several years in implementing these methods in our studies. First, the peripheral blood mononuclear cells (PBMCs) will be isolated by the Functional Genomics Laboratory using a previously published protocol through centrifugation, washing, and reduction of the supernatant.[17] *Monocytes* will be separated from the resultant mononuclear cell suspension using a commercially available kit, the Monocyte Isolation Kit II (Miltenyi Biotec Inc., Auburn, CA). Briefly, the Monocyte Isolation Kit II is an indirect magnetic labeling system for the isolation of untouched monocytes from human PBMCs. Non-monocytes, such as T cells, NK cells, B cells, dendritic cells, and basophils, are indirectly magnetically labeled using a cocktail of biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123, and CD235a (Glycophorin A), as well as Anti-Biotin MicroBeads. Highly pure unlabeled monocytes are obtained by depletion of the labeled cells and pelleted using gentle centrifugation, for the gene expression studies described below. Then, the magnetically labeled non-monocytic cells (chiefly lymphocytes) will be isolated according to the manufacturer's established method for use in the DNA methylation study, as described below. The isolated samples of monocytic RNA (for expression studies) and non-monocytic DNA (for methylation studies) will be stored at -70°C for batched analysis.

Sample Analysis

Endothelial Activation. We measure plasma *endothelin-1* using ELISA techniques (R&D Systems, Minneapolis).

Lipid Peroxidation. The measurement of plasma *malondialdehyde* (MDA) concentration, a marker of lipid peroxidation, will be assessed using a method based (Bioxytech, Oxis International, Portland, OR) on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1, NMPI), with MDA at 45°C in HCL with Probucol to minimize the reaction of 4-hydroxyalkenals. This technique provides estimates for total MDA that are similar to levels obtained using HPLC. [41-45] The measurement of *oxidized LDL* is by means of an monoclonal antibody–based ELISA (Mercodia, Uppsala, Sweden). mAb-4E6 is directed against a conformational epitope in the apolipoprotein B-100 moiety of LDL that is generated as a consequence of the substitution of 60 lysine residues of apolipoprotein B-100 with aldehydes.

Response to phospholipid oxidation. Antibodies that recognize oxidized phopholipid will be measured by Dr. Rosenfeld's lab (as in Project 3) in the serum according to the method of Rolla et al.[46] Briefly, phospholipids are oxidized with 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH; Polyscience Inc.,Warrington, PA). Cardiolipin, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol is then suspended in PBS and incubated for 6 h at 37°C in the presence of 1 mmol/l AAPH. Oxidized phospholipids are extracted with chloroform-methanol (1:1 v/v), dried under N_2 and resuspended at 100 mg/ml in ethanol. Antibody binding to oxidized phospholipids is determined by solid-phase immunoassay. Non-specific binding sites are blocked by incubation with 1% (v/v) PEG in PBS pH 7·4. The diluted human sera is then added and incubated for 1 h at 37°C. Peroxidase-linked goat anti-human IgG or IgM (Dako) is then added and incubated for 60 min at 37°C. Antibody

binding is measured by the addition of a reaction mixture containing 0·4 mg/ml of 1-phenylenediamine, 0·4 ml/ml hydrogen peroxide (30%), 5·1 mg/ml citric acid, 6·1 mg/ml anhydrous Na2HPO4, pH 5·0. The reaction is stopped after 15 min by adding 50 ml 2N H2SO4 and absorbance is measured at 490 nm.

Gene Expression Approach

RNA isolation and storage methods. Monocytic RNA will be isolated and stored by the CEEH Functional Genomics Laboratory according to our previously describe methods.[17] Total RNA is isolated from the buffy coat using the Trizol method. RNA concentration is determined using ultraviolet (UV) spectrophotometry, and quality is assessed using the Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Quantitative RT-PCR (qRT-PCR) using fluorogenic 5' nuclease-based assays. To characterize the levels of expression of specific genes, the CEEH Functional Genomics Laboratory will develop these assays and then, use an ABI 7900 Sequence Detection System that allows quantitation of specific mRNA levels using fluorogenic 5' nuclease-based assays. These methods are well established in this laboratory and have been previously described.[47-50]

DNA Methylation in Circulating Lymphocytes

DNA methylation status will be measured using the Methyl-Profiler PCR Array System (SA Biosciences, Frederick, MD). This system detects DNA methylation status at the CpG islands through differential cleavage of target sequences by two different restriction endonucleases. These enzymes require either the presence or absence of methylated cytosines in their respective recognition sites. DNA will be isolated from PBMCs in the buffy coat cell samples (after removal of the monocytes for gene expression studies) using the QIAmp DNA mini kit (Qiagen, Valencia, CA) for these methylation studies. Real-time PCR is used to quantify the relative amounts of methylated and non-methylated DNA remaining after enzyme digestions. These measurements will be done in the Functional Genomics Laboratory using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems Inc., Hercules, CA). This process replaces the bisulfite conversion process, which can damage the DNA and can lead to spurious results.

Safety Measures

To determine proper engine operation and detect unanticipated exposure problems, we monitor CO and NO_2 continuously throughout the exposures, as well as numerous engine and exposure parameters. Through a window, a technician observes the subject throughout the exposure period. There is continuous monitoring of pulse, O_2 saturation, cardiac rhythm, and blood pressure in the subjects. Resuscitation equipment is available in the exposure facility as well as at all testing locations.

Anticipated Results

Aim 1: The primary analysis will use the robust crossover design to independently determine the comparative effects of the two pollutant atmospheres and the ALOX15 G189C status on the vascular response to traffic-related pollutants in human subjects.

We hypothesize that the responses of vasoconstriction, plasma endothelin-1, and systolic blood pressure will be significantly greater with the HP pollutant atmosphere compared to either the LP pollutant atmosphere or the filtered air exposure, while the LP and FA responses will be not different. *(Aim 1a)*

We also hypothesize that presence of the G allele of the ALOX15 G189C SNP will be associated with increased vasoconstrictive response to HP pollutant (compared to filtered air), and increased BP response to HP pollutant, compared to the A allele, in an additive model. *(Aim 1b)*

Based on our preliminary data we do not anticipate problems with logistics (e.g., subject recruitment, availability of subjects with varying ALOX15 G189C, status, ability to maintain exposure concentrations, ability to test subjects through five conditions, etc).

Aim 2: In this aim we are addressing biological mechanisms that underlie the observed vascular effects of traffic-related air pollutants, and providing insight into acute effects that translate into chronic sequelae including atherogenesis. Since the relationship between surfactant oxidation in the pulmonary system (as addressed in Project 3), lipid peroxidation, inflammation, and endothelial activation is complex and multi-directional, the impact of our interventions is difficult to predict, but we are able to establish *a priori* hypotheses for testing based on our preliminary data and findings in the literature.

Based on available information and nonhuman models, we anticipate that we will observe an increase in anti-phospholipid antibodies, malondialdehyde and oxidized LDL *(Aim 2a)*, as well as modified gene transcription (increase in monocyte RNA expression of HMOX1, decrease in GCLC and PPARA) *(Aim 2b)*, with the HP pollutant exposure compared to the FA exposure. This will support an overarching hypothesis of upstream surfactant oxidation in the airways, lipid peroxidation in the circulation, and initiation of downstream pathways exerting vascular tissue level oxidative stress and atherosclerosis.

We anticipate that the monocyte gene expression effects of the pollutant exposure will be blocked or blunted by the lipoic acid intervention. *(Aim 2c)* (We anticipate that the lipoic acid intervention will provide an overwhelming effect on the plasma markers so do not anticipate being able to clearly observe the interaction with pollutant exposures for these outcomes.) This will serve to test the importance of lipid peroxidation in the propagation of signaling pathways through the immune system, as demonstrated in the monocyte.

Finally, we anticipate that the HP pollutant exposure will increase hypermethylation of FOXP3 in lymphocytes, compared to filtered air, implying a gene-silencing effect on development of the anti-atherogenic Tregs.

Taken together, this experimental design provides a suite of complementary hypotheses to be tested that provide insight both into early effects of traffic-related air pollutants on the vasculature (important in *triggering* of cardiovascular events*)* as well as how these exposures can initiate and propagate long-term sequalae (such as accelerating the progression of atherosclerosis).

Data Analysis and Statistical Considerations

In this crossover study, each subject is observed for a sequence of five periods. Each period is separated by a wash-out period to reduce the likelihood of carry-over effects. Attention to the potential for carry-over and period effects is also addressed in the analysis.

For all outcomes, we conduct a paired analysis to take advantage of the within-individual crossover design, and also adjust for intra-individual variation by assessing the difference from pre-exposure to post-exposure measurements, and then compare that difference between the control day (e.g, filtered air) and the treatment day (e.g., the HP exposure).

Statistical analyses will proceed in three phases: descriptive, hypothesis testing, and exploratory. Initially we will perform descriptive data analyses on all the data. These will include summary statistics (mean, median), assessment of normality (e.g., Q-Q plots), graphical displays and estimates of correlation. Hypothesis testing will be limited to the primary outcomes defined *a priori* (e.g., exposure related changes in brachial artery diameter, endothelin-1 concentration, and blood pressure for Aim 1). We generally compare the difference between pre-exposure and post-exposure results between the filtered air and specific exposure situations in a paired approach. Crude tests of the primary outcomes (e.g. paired t-tests and ANOVA) will provide a transition from the descriptive to the hypothesis testing phases of the analysis. These will precede fitting of generalized linear models that incorporate the crossover design features, and impact of pharmacologic intervention or genotypic variation. For the continuous outcomes in experiments, we will assume the outcome (or its log-transformation) is normally distributed. If this assumption is incorrect, non-parametric alternatives will be considered.[51]

Table 2. Exposure groups.

	Lipoic Acid	*Placebo*
High Potency Atmosphere	A	B
Low Potency Atmosphere		C
Filtered Air	D	E

We can operationalize the planned comparisons by way of a table of the exposure groups, as shown here.

Aim 1a will compare the exposure effect of brachial artery diameter (after exposure vs. before exposure), systolic blood pressure (at one hour of exposure vs. before exposure), and plasma endothelin -1 concentrations (3 hrs post exposure initiation vs. before exposure) in session B to that found in both exposures C and E.

Aim 1b will compare the systolic blood pressure and brachial artery diameter response in session B to that in exposure E, and test for effect of an interaction term provided by the number of G alleles at rs2664593. Endothelin-1 effects will be considered a secondary hypothesis for this sub-aim.

Aim 2a will compare the differences in concentrations of malondialdehyde (3 hours post exposure initiation vs. before exposure), anti-phospholipid antibodies (next day vs. before exposure), and plasma oxidized LDL (next day vs. before exposure) in session B to that found in exposure E. If those differences are significant, we will assess whether session C is different from either session B or session E.

Aim 2b will assess whether fold-differences (3 hours post exposure initiation vs. before exposure) in monocyte mRNA concentrations are different in session B compared to session E.

Aim 2c will assess whether the change in fold-differences in monocyte mRNA concentrations between sessions A and D is equal to the change between sessions B and E.

Aim 2d will assess whether there is an increase (next day compared to pre-exposure) in percent of FOXP3 regions characterized as hypermethylated in session B compared to session E.

Sample Size

We have had excellent results observing statistically and clinically significant health effects with sample sizes of between 20 and 25 participants in the vascular outcomes. We propose 24 participants in this study as it simplifies balancing of our randomization approach. We have already demonstrated that in the proposed experiment we will have sufficient statistical power (comparing high-toxicity exposure to filtered air, and high toxicity to low-toxicity exposures) to determine effects in the vasoconstriction, endothelin, DNA methylation in FOXP3, and in the

gene expression aims (if we use a hypothesis-testing approach for these three genes rather than a shotgun array approach). We have not previously assessed the outcome of malondialdehyde and oxidized LDL, so power calculations for those are provided here. Insufficient data is available on the anti-phospolipid antibody assays in humans to conduct power calculations, so those studies will be considered exploratory at this time.

For malondialdehyde, we assess power to detect an effect of one-third the magnitude by smoking cessation.[52] The probability is 85 percent that the study will detect a treatment difference at a two sided 5 percent significance level, if the true difference between the treatments is 0.077 micro-molar concentration. This is based on the assumption that the standard deviation of the difference in the response variables is 0.120, which is conservative since it represents the cross-sectional rather than within-individual variance. Using a similar approach, we have 90% power to detect an effect one-fourth the magnitude (in opposite direction) of the impact of low dose Vitamin E on oxidized LDL.[53] Of note, those observations for oxLDL were noted in less than 200 minutes from time of supplementation.

3. Expected Results, Benefits, Outputs, and Outcomes:

By coordinating closely with Center Projects 1-3, we will determine whether specific aspects of traffic-derived exposure (primary vs. secondary organics, particulate vs. gases, spark-ignition vs. diesel engine vs. a mixture) enhance the human vascular response to pollutants. We also will learn about biological mechanisms involved in human health effects from air pollutants. It translates current molecular and cellular knowledge, and evolving understanding of both air pollutant health effects and cardiovascular pathophysiology, into hypotheses that can be directly tested in human subjects. By assessing human effects of an exposure directly relevant to ambient sources of air pollution, at exposure levels less than an order of magnitude greater than urban peak exposures, we will provide information directly relevant to risk assessment. These studies will also provide new approaches for the prevention of cardiovascular health effects.

4. General Project Information:

Joel Kaufman serves as director of the project, with local exposure generation and exposure characterization directed by Professor Larson, and Dr. MacDonald providing the leadership from LRRI to create the exposures to mimic those at their laboratory.

Clinical space is available both at the DEOHS Roosevelt building, where Dr. Kaufman's other labs are based, as well as at the UW Medical Center General Clinical Research Center.

The Functional Genomics Laboratory, directed by Dr. Fred Farin, is adjacent to Dr. Kaufman's lab, and has a long history of supporting DEOHS investigators, including Dr. Kaufman, in use of state-of-the-art technologies. This laboratory has a full-suite of "-omics" technological capability including Affymetrix platform, ABI sequencing equipment, BioRad multiplex capability, and well-trained lab personnel. Through the NIEHS Center, these services can be accessed at reduced cost.

Project 5: Effects of Long-Term Exposure to Traffic-Derived Particles and Gases on Subclinical Measures of Cardiovascular Disease in a Multi-Ethnic Cohort

The overarching goal of the UW CCAR is to better define the impacts of roadway-source air pollutants on cardiovascular disease morbidity. We propose an integrated suite of studies utilizing state-of-the-art characterization of multiple near-roadway pollutant concentrations to inform both the development of laboratory-generated atmospheres for animal and human exposure studies, as well as to develop multi-pollutant exposure models for epidemiologic analyses. Project 5 provides this epidemiologic component and will allow us to transform the existing Multi-Ethnic Study of Atherosclerosis and Air Pollution (MESA Air) from its current focus on $PM_{2.5}$ into a multi-pollutant study, which can meaningfully investigate the impact of traffic-derived air pollution on cardiovascular health using a source-to-exposure approach.

OBJECTIVES

Objective 1: To build a multi-pollutant exposure model for traffic-derived air pollutants, incorporating complex spatial information on primary and secondary traffic-derived particles and gases for use in epidemiological analysis.

Objective 1A. To develop an exposure surface for traffic-derived air pollution, employing the small-scale gradient data acquired as part of the mobile monitoring campaign in Project 1. In order to predict the multi-pollutant spatial field of long-term average ambient concentrations, these data will be integrated with data from a central fixed site operating during the monitoring campaign, regulatory monitoring data, and geographic covariates to construct a land-use regression-based universal co-kriging multivariate prediction model.

Objective 1B. To further utilize the mobile monitoring data from Project 1 to develop distributions of on-road pollutant concentration estimates for various roadway types and traffic conditions in each study city.

Objective 2: To develop and validate individual-level exposure estimates for traffic-derived air pollutants, including a determination of the effect of time in transit on these estimates.

Objective 2A: To better understand the in-transit component of total exposure using a personal monitoring approach. We will recruit 144 members of the MESA Air cohort to participate in two-week residential, personal, and in-vehicle monitoring campaigns in each of two seasons. This monitoring will also include a GPS data-logging component to refine the on-roadway exposure estimates and to verify the travel habits as reported by questionnaire.

Objective 2B: To develop individual-level predictions of exposure to traffic-derived air pollutants, integrating: 1) the outdoor residential concentration estimates from Objective 1A; 2) estimates of residential infiltration rates; 3) road class- and traffic condition-specific estimates of on-roadway concentrations from Objective 1B; and 4) individual-level, questionnaire-derived time-location information. The personal monitoring data from Objective 2A will be used to understand the measurement error associated with these predictions.

Objective 3: To estimate the effect of individual-level exposure to traffic-derived air pollution on subclinical cardiovascular disease in the MESA Air cohort, using exposure models derived from Objective 1 and incorporating time-location data from Objective 2. This analysis of the effects of individual exposure to traffic-related pollutants will incorporate recently developed methods to correct for the measurement error resulting from the use of

predicted exposures. We will also apply a novel statistical approach, developed in the Biostatistics Core, to estimate the relative health effects of pollutant mixtures.

Objective 3A: To understand the relation between exposure to traffic-derived air pollutants and change in left ventricular myocardial mass over 10 years, assessed via magnetic resonance imaging (MRI).

Objective 3B: To understand the impact of exposure to traffic-derived air pollutants on the microvasculature, based on arteriolar diameters measured repeatedly by retinal photography over eight years.

Objective 3C: To evaluate the performance of our refined exposure estimates for traffic-derived air pollutants relative to the existing metrics developed in MESA Air for the primary MESA Air outcomes of subclinical atherosclerosis progression and incidence of CVD events.

Objective 3D: To understand the impact of exposure to traffic-derived air pollutants on DNA methylation.

BACKGROUND

A substantial body of evidence links exposure to air pollution with cardiovascular morbidity and mortality; cardiovascular disease explains most of the excess mortality from air pollution.[1,2] Fine particulate matter ($PM_{2.5}$) has been particularly implicated. Although the specific sources and chemical and physical features of air pollution most responsible for these cardiovascular effects are not yet known, there is both experimental and observational evidence that supports a role for emissions from traffic or roadways.[2]

Most studies implicating traffic effects have relied on roadway proximity or crude surrogates. Near-roadway pollution is a complex mixture of particle and gas phase components that vary by vehicle emission source, road surface, extent of physical aging and the type and degree of atmospheric processing and photochemical reactions.[3] There is currently no air pollutant or pollution component that serves as a specific measure of traffic pollution. Ambient air concentrations of pollutants such as nitrogen dioxide (NO_2) and carbon monoxide (CO) are often used to reflect traffic pollution, but there are other sources of these pollutants. Concentration of particulate matter elemental carbon (EC, or black carbon) is often used to reflect diesel vehicle emissions specifically, but in many settings a substantial fraction of ambient EC is attributable to non-diesel or even non-traffic emissions.[4]

In epidemiologic investigations of long-term effects of exposure to traffic-related pollutants, an alternative to estimates based on measured pollutant concentrations has been to approximate exposure as the magnitude of traffic itself at relatively small spatial scales (e.g., tens of meters). Examples of these exposure measures include proximity to large roadways, length of roads or traffic volume within a defined distance, or emissions based on dispersion models.[2,5-7] Latent variable regression, which makes use of monitored pollutant concentrations, has also been used to estimate long-term exposure at larger spatial scales by attempting to isolate the part of these pollutants due to traffic emissions.[8,9] However, none of these approaches to estimating exposure to traffic pollutants in an epidemiological study has provided insight into the specific characteristics or components of traffic pollution that are responsible for the effects observed. While distance from roadway or absolute traffic volume provides individual-level exposure estimates, the non-specificity of these estimates limits their utility in identifying the characteristics of the near roadway mix that are more critical determinants of health outcomes.

Recently, stationary monitoring campaigns have made improvements in their ability to characterize primary and secondary aerosol features due to traffic exposures (e.g., Pabkin et al. 2009),[10] but methods have not been developed to extend these over larger geographic surfaces or implement them in epidemiological research. We have devised a novel approach to incorporate traffic-derived pollutant exposures into epidemiological analyses using a combination of mobile and fixed monitoring, and propose to implement it in an ongoing large-scale cohort study. Mobile monitoring is an alternative to both fixed site monitoring and to the distance/traffic volume measures of near roadway exposure. Estimation of long-term exposure effects, particularly for pollutants that exhibit substantial spatial variability, requires long-term measurements at multiple locations. Land use regression, used in concert with long-term fixed site measurements, has similar requirements for a large number of monitoring locations. Mobile monitoring, as we propose to use here, and as described in more detail in Project 1, can overcome these limitations in characterizing spatial variability; when used together with land use regression techniques, relatively short periods of mobile monitoring can be used to estimate pollutant concentrations over fine spatial scales.[11]

This proposed project will build upon the Multi-Ethnic Study of Atherosclerosis and Air Pollution ("MESA Air", PI: Dr. Kaufman, RD831697), a STAR research project funded in 2004 to examine the long-term health effects of $PM_{2.5}$. MESA Air is a prospective cohort study, anchored on the NIH/NHLBI Multi-Ethnic Study of Atherosclerosis (MESA). MESA was initiated in July 2000 to investigate the prevalence, correlates, and progression of subclinical cardiovascular disease.[12] By adding detailed exposure assessment for $PM_{2.5}$ and expanding the exposure heterogeneity in the cohort, MESA Air is assessing the relation between individual-level estimates of long-term $PM_{2.5}$ exposures, subclinical progression of atherosclerosis (primarily assessed using coronary artery calcification, measured via CT, and intima-media thickness, measured via ultrasound), and incidence of cardiovascular events. MESA Air is anticipated to generate findings of regulatory importance, as it has more complete individual information on potentially confounding cardiovascular risk factors, has better measured outcome measures (especially than studies that rely on death certificate data), and uses a more refined individual-level exposure assignment for airborne particles and gases than any long-term air pollution cohort study to date. MESA Air is focused primarily on $PM_{2.5}$, and on understanding how ambient-derived $PM_{2.5}$ exposures relate to cardiovascular health. Though a number of pollutants are measured in MESA Air, it is not designed around a multi-pollutant model, nor does it focus specifically on traffic-derived pollution or assess the impact of time-in-transit on total exposure.

Micro-environmental impact of traffic: The special case of on-road exposures

On-road exposures have been largely neglected in long-term epidemiological studies of air pollution, even those incorporating traffic-related exposure metrics. Because on-road concentrations of some pollutants can be dramatically higher than concentrations even a short distance from a major roadway, cumulative exposures to traffic-generated pollutants could potentially be dominated by time spent commuting or in traffic. Exposure estimates that do not account for this potentially dominant exposure could be in serious error. As part of this study, we will obtain in-transit, on-road concentration measurements of traffic-related pollutants using our mobile monitoring platform, and validate these estimates using a personal monitoring campaign. With these data, we will determine whether incorporation of on-road data has a meaningful effect on exposure estimates that do not make use of such data; if the exposure

estimates are different, we will then determine whether the exposure estimates that incorporate on-road data result in different health effect estimates.

From preliminary personal monitoring data in MESA Air, we suspect that the impact of time-in-transit on total exposure may be an important component of total individual-level exposure. Personal monitoring was included as one of the campaigns within the MESA Air exposure monitoring framework. Ninety (90) participants wore backpack-mounted passive and active monitoring equipment to determine their personal exposure to $PM_{2.5}$ and NO_x. Concurrent indoor and outdoor residential monitoring was conducted with the personal monitoring. Individual-level exposure *predictions*, based solely on the concurrent residential indoor and outdoor measurements, were found to correspond well with the personal monitoring *measurements* (median relative percent difference ~10%). However, while the correspondence between measured and predicted personal exposure increased with increasing self-report of time spent at home, this correspondence was reduced with increasing time spent in transit. People who reported spending more time in transit had larger percent differences between measured and predicted exposures than those who reported less. The percent difference was approximately 2-fold higher for those in the highest quartile of time spent in traffic compared to the lowest. Hence, capturing exposures due to time in traffic could be an important component of total personal exposure for people with significant travel time.

Subclinical Cardiovascular Disease Outcomes

The endpoints chosen for this study were selected based on strong associations recently found in MESA Air, and are described in more detail below.

Proximity to Traffic is Associated with Increased LV Mass

Prior research has supported the finding of increased cardiovascular disease associated with residential proximity to roadways.[13-15] Left ventricular mass index (LVMI) provides important evidence of end-organ cardiac damage and confers increased risk for development of MI and congestive heart failure.[16,17] Using data from MESA, we analyzed MRI measurement of LVMI in 2177 participants. After adjusting for gender, body surface area, age, race, current smoking, education, income, current alcohol consumption, area, and systolic blood pressure, and relative to participants living more than 150 m from a major roadway, participants living within 50 m of a major roadway showed 1.4 g/m^2 (95% CI, 0.3-2.5) higher LVMI, a difference in mass corresponding to a 5.6 mm Hg greater systolic blood pressure.[7]

Impact of Air Pollution on Microvascular Circulation

In addition to the more easily observed effects on conduit arteries and resistance vessels, an important effect of air pollution is on the microvascular circulation. Nurkiewicz and colleagues have demonstrated that ultrafine particles and diesel exhaust constituents interfere with normal microvascular function.[18-24] Recent *in vitro* studies of human microvascular endothelial cells[25,26] have provided additional mechanistic insight into air pollution toxicity, indicating that ultrafine particles can directly stimulate these cells to generate reactive oxygen species via activation of NADPH oxidase. Also, it seems most likely that the acute ischemic effects observed during exercise and diesel exhaust exposure in men with coronary artery disease by Mills [27] represents a microvascular phenomenon rather than vasoconstriction of the epicardial coronary arteries.

We have begun assessing the impact of air pollution on the microvasculature within MESA Air, examining cross-sectional associations between MESA Air estimates of long-term exposures to air pollution and average retinal vessel diameters. Vessel diameters were assessed using digital

retinal images taken in 4,408 MESA participants between 2002 and 2003 and expressed by the corrected retinal arteriolar equivalent (CRAE); regional-level air pollution exposures were assigned for each participant using annual average outdoor residential $PM_{2.5}$ concentrations. As hypothesized, retinal arteriolar diameter was reduced in response to increases in both long- and short-term estimates of $PM_{2.5}$ concentrations. These relationships were strong, with 0.9 μm (95% CI: -1.2 to -0.5) and 0.4 μm (95% CI: -0.8 to -0.1) decreases in CRAE per inter-quartile increases in long- (3 $\mu g/m^3$) and short-term (10 $\mu g/m^3$) $PM_{2.5}$ levels, respectively.

Epigenetics May Provide Insight into Cardiovascular Effects of Air Pollution

It has been demonstrated (in animal models, at least) that certain environmental exposures can interact with specific DNA sequences to produce persistent epigenetic modifications that can influence gene expression and presumably susceptibility to disease.[28] Most attention to epigenetic research has been focused on early life exposure until some recent studies from Baccarelli and colleagues, who demonstrated changes in "global" DNA methylation (long interspersed nucleotide element (LINE)-1 and Alu repetitive elements) with relatively small daily-scale exposures to ambient traffic-related air pollutants and decreased iNOS promoter DNA methylation with (larger) particulate exposure excursions in steelworkers.[29,30] Data developed in our own laboratory (see also Project 4), in which DNA methylation assays were performed for 5 subjects before and after controlled exposure to diesel exhaust and filtered air exposures has also demonstrated the potential of using this approach to understand these health effects. We found that, in the FOXP3 gene, the proportion of regions becoming hypermethylated was elevated after diesel exhaust exposure, relative to after the fresh-air control (mean difference in morning-after-exposure vs. before-exposure percent hypermethylated: 7.1%, $p = 0.03$.)

STUDY APPROACH

Approach to Objective 1A: Individual level outdoor exposure estimates.

We will utilize data from the mobile monitoring campaign in Project 1, Objective 1 to predict a multi-pollutant spatial surface of annual average concentrations at subject homes. We will develop a temporally adjusted universal co-kriging model in order to account for dependence of the concentrations on geographic covariates as well as correlation across space and between pollutants. The model will be developed by the Biostatistics Core (see Section 3.a.3).

As described in Project 1, Objective 1, mobile monitoring will be conducted in four MESA Air cities: Baltimore, Winston-Salem, Chicago, and Los Angeles, at approximately 40 locations in each city selected to represent different strata of ambient NO_x. The multi-pollutant vector of observations will include several gases, measures of particulate matter, and particle bound PAHs. Data will be collected in 15 second segments in the afternoon and evening at 40 cloverleaf locations over the course of three days, and this entire procedure will be repeated three times at the same set of locations for a total of nine days of sampling within each of two seasons. Approximately 15 cloverleafs will be sampled each day, allowing for 3 of the 40 locations to be sampled every day. The 14 routes between cloverleafs will also be monitored, resulting in a total of 15+14=29 paths per day of sampling. A fixed site will also be located in each city during the mobile monitoring campaign.

In order to account for temporal trends during the sampling period, the mobile data will be normalized based on measurements at the fixed site:

$$C_{15\,\mathrm{sec}}^{adjusted} = C_{15\,\mathrm{sec}}^{mobile} \left\{ \frac{C_{\mathrm{overall}}^{fixed}}{C_{30\,\mathrm{min}}^{fixed}} \right\}.$$

The subscripts refer to the signal averaging time. The 30-minute fixed site average is centered on the 15 second mobile monitoring measurement interval, and the overall average is for all measurements taken at the fixed site during the season's sampling campaign. This normalization is designed to remove temporal trends within the sampling period so that $C_{15\,\mathrm{sec}}^{adjusted}$ can be regarded as a purely spatial field at locations along the sampling path (so each $C_{15\,\mathrm{sec}}^{adjusted}$ represents the time-normalized concentration on the short road segment traversed during the 15 second interval). This method of adjustment is based on the assumption that the temporal trend within each sampling period is spatially uniform. This assumption will be evaluated by examining the temporal structure of the adjusted concentrations for each pollutant at the three cloverleafs that are sampled every day. If we find that it is necessary to introduce a more flexible temporal adjustment, we will adapt the spatio-temporal model developed in MESA Air[31] to predict spatially varying temporal trends at the hourly time scale for NO_x and other pollutants for which there is sufficient monitoring data from the EPA's regulatory network. We will estimate spatially varying temporal trends for other pollutants by taking appropriate linear combinations of the trends estimated for pollutants with rich regulatory data.

After adjustment for temporal trends and log-transformation, for each pollutant *q* we have spatial concentration data of the form X_{qlkr}^{s} for coordinates *s* along path *k* (either a cloverleaf or the path between two cloverleafs) in city *l* during season *r*. Although we do not index time because the temporal pattern has been removed, each measurement corresponds to a 15 s period. In order to make predictions at subject home locations and other locations without measured data, we will exploit the relationship between concentrations and geographic (land use) covariates S_{lkj}^{s} for *j=1,…,J*. The set of covariates we consider will include traffic density in various buffers, population density, land use, distance to coast, and roadway census feature class codes (CFFC).

We will reduce the dimension of the monitoring data by taking the median over *s* to obtain X_{qlkr}, an approximation to average concentration over path *k* that is robust to outliers from very short term variations in traffic or meteorology. We will obtain corresponding average geographic covariates S_{lkj} by taking the median value over *s*. For each pollutant, site, and season, we construct a universal kriging model with a land-use regression (LUR) mean field based on a subset of geographic covariates. In order to minimize multicollinearity that can lead to overfitting, we select an optimal subset of geographic covariates with an algorithm similar to a method recently developed for LUR,[32] adapted to universal kriging by basing the univariate correlation scores on leave-one-out cross-validation. The geographic location of X_{qlkr} is based on the centroid of path *k*; the model allows for different nuggets for each measurement based on the length of path *k*.

Since we expect the various pollutants to be correlated, after we have selected covariates in individual universal kriging models, we will construct a unified co-kriging prediction model based on a linear model of co-regionalization. This approach to co-kriging allows for each pollution component to have its own marginal covariance structure.[33] Additional details are given in the Approach to Objective 3 and in the Biostatistics Core section of this proposal.

We will predict long-term average multi-pollutant concentration fields by combining the predictions from the two seasonal co-kriging models in each city. We will evaluate the quality of these predictions by comparing them to outdoor passive monitoring data collected in Project 1 and Project 5 and to annual averages of data from regulatory monitors. If the agreement is poor, we will examine the residuals to determine if there is evidence of spatially varying seasonality. If so, we will modify our predicted annual averages based on seasonal trends estimated for a subset of pollutants by applying the MESA Air spatio-temporal model[31] to regulatory and MESA Air monitoring data. As an exploratory analysis, we will also consider excluding the paths between cloverleafs, which may be more affected by short-term variations in wind direction.

Approach to Objective 1B: Estimates of on-roadway exposures.

We will use the mobile monitoring data and a universal co-kriging model analogous to the one in Objective 1A to predict the impact of traffic patterns and roadway class on pollutant concentrations. This will primarily involve including as geographic covariates the CFCC of the road the mobile monitor is driving on and an estimate of traffic conditions based on its speed as measured by an on-board GPS unit. We will also modify the definition of paths to separate time periods when the mobile monitor is traveling on high and low traffic roads as well as different road conditions. Since the geographic covariates are averaged over each path, this will provide increased contrast between pre-averaged measurements and will result in improved predictions.

Approach to Objective 2A: Personal and in-vehicle monitoring.

We propose an integrated personal, residential, and in-vehicle monitoring campaign to better understand the in-transit component of total exposure. As part of this objective, we will estimate each participant's exposure to traffic-related pollutants based on personal monitoring data. We will also estimate individual-level exposures using a combination of measured indoor, outdoor, and in-transit exposures, weighted by time spent in each location as measured by GPS. These measures will also be compared to predicted exposures using modeled data (see Objective 2B). We will evaluate the relationships among these three measures (e.g., personal monitoring measurements; predicted exposures based on indoor, outdoor and in-transit measurements; and predicted exposures based on modeled exposures), and will determine how factors such as time spent in traffic or road type traveled impact that correspondence.

For this monitoring campaign, will recruit 72 subjects from each of two cities, LA and Chicago (total n=144). These participants will be selected based on previous agreement to participate in personal monitoring and will be chosen specifically to include those with a range of reported commuting times. The indoor, outdoor, and personal samplers will be deployed for two-weeks while the in-vehicle sampler will monitor only during the times when the participants are in transit in a vehicle within those two weeks. All participants will record their locations on a time-activity diary and carry with them a GPS data-logging location tracker throughout the study.

<u>Monitoring methodology</u>

In each sampling location, we will collect time-integrated measures of NO, NO_2, NO_X, SO_2, and O_3 using two different configurations of the Ogawa passive sampler (one two-sided sampler for NO_x/ NO_2/ SO_2 and a second single sided sampler for O_3). We will also collect time-integrated measures of a suite of 11 VOCs: benzene, isoprene, toluene, n-decane, n-nonane, 2-methylpentane, m-xylene, undecane, i-pentane, n-pentane, and o-xylene, using the 3M 3520 Organic Vapor Monitor (OVM). VOC selection was based on previous studies identifying the

most significant and distinguishable markers of fresh and aged gasoline emissions, diesel emissions, and evaporative emissions in PMF and UNMIX receptor modeling.[34-37] The selection process for these compounds was described previously in Project 1, Table 3.

Participants will clip the personal monitoring badges to their shirt or keep setup near them throughout the 14-day sampling period. In addition, a stand fitted with each sampler type will be set up inside and outside of each subject's home. For the in-vehicle monitoring, a novel sampling platform has been designed to mount multiple passive monitoring badges and record their exposure durations. This small platform (9" x 12" x 4") consists of a circular mounting frame, a sealable protective enclosure, and a small electronics box. The passive badges are mounted in a circular array and remain in an airtight enclosure until sampling is required during travel. Solid inserts are placed within the mounting frame to minimize dead air space. At the start of a given trip, the participant removes the cover and touches a timer switch; at the end of trip, they replace the cover and switch the unit off. A temperature and humidity monitor will log data continuously throughout the 2-week period. To minimize interference from the vehicle's HVAC system and to sample in the most representative location within the vehicle, the platform will be mounted vertically on the passenger seat at breathing height. If multiple passengers are in the vehicle, the platform can be moved to the back of the passenger seat.

We will deploy and collect 10% duplicate field samples and 10% field blanks, to assess the precision and limit of detection (LOD) of each of the three samplers and the associated methodologies. Samplers will be cold shipped from the UW laboratory to the field laboratory and deployed by field technicians. The Ogawa monitors will be analyzed at the UW by ion chromatography (IC) to measure NO_2, SO_2, and O_3, and by ultraviolet spectroscopy (UV), for NO_x. The 3M OVM 3520 is analyzed by GC/MS in conjunction with a Programmed Temperature Vaporization (PTV) Injector for improved sensitivity.

Based on ambient levels of VOCs in Cook County (Chicago) and Los Angeles,[38] we anticipate ambient levels of our suite of VOCs to be in the 0.06 (undecane) to 4.1 $\mu g/m^3$ (toluene) range. Using the manufacturer's reported collection rates for these VOCs, the lower limit of the net collected mass will be in the 0.023 (undecane) to 0.99 μg (n-pentane) range, which is above the LOD for the GC/MS analysis. In-vehicle exposures will be of shorter duration, but the levels are anticipated to be significantly higher than ambient. Based on previous studies,[39-41] we anticipate concentrations in the 0.20 (n-nonane) to 11.8 $\mu g/m^3$ (toluene) and higher range, which would provide for filter loading above our LOD. The Ogawa sampler has an LOD of 0.3, 0.5, and 0.4 ppb for NO_2/ NO_x, SO_2, and O_3 respectively, for 2-week duration samples.[42-44] To accommodate the shorter sampling period of the car sampler, we are using an array of three samplers for NO_2, NO_x, SO_2 and O_3, which will provide an LOD of 1.3, 2.2, and 1.5 ppb respectively for the in-transit samplers based on our estimated mean exposure duration.

Participant location data will be logged in a cell phone enabled with a custom application written on the Google Android framework. Android operates on a Linux kernel, allowing applications to run in the background, such that location is recorded automatically. The application will "wake up" every 5 minutes, access the location service supported by the device to obtain global position, and record the data in a table on the SQLite database provided through Android.

Approach to Objective 2B: Individual-level exposure predictions.

For each MESA Air participant in the four cities included in this project (n=4,239), we will develop individual-level estimates of exposure to traffic-derived particles and gases, integrating: 1) the outdoor residential concentration estimates from Objective 1A; 2) estimates of residential infiltration rates (see below), and 3) road class- and traffic condition-specific estimates of on-roadway concentrations from Objective 1B. These components will be time-weighted using individual-level questionnaire-derived time location information according to:

$$c_i = t_i^{(\text{outdoor})} c_i^{(\text{home})} + t_i^{(\text{indoor})} c_i^{(\text{home})} d_i + t_i^{(\text{transit})} c_i^{(\text{transit})}$$

where i is the subject index, c_i is the total individual-level exposure to traffic-derived pollutants, $c_i^{(home)}$ is the residential outdoor exposure estimate, $c_i^{(transit)}$ is the on-roadway exposure estimate, t is the fraction of time per week spent in each location, and d_i is the infiltration efficiency.

In order to estimate infiltration efficiencies of traffic-related black carbon at all subject homes, we will use our previous estimates of the infiltration efficiencies based on particulate sulfur at these same homes.[45] These latter values will be adjusted by a ratio of the theoretical infiltration efficiency for black carbon to that for sulfur. The theoretical values will be computed based on previously established literature values of penetration and loss rate as a function of particle size[46,47] assuming the same air exchange rate for each compound. The size distribution of regional sulfate is well established and corresponding penetration and loss rates are not very sensitive in this size range. The assumed black carbon size distribution will be checked against the relationship between black carbon and particle size observed via the mobile platform.

To understand VOC infiltration, we first estimate individual level VOC exposure for subjects for whom we have separate measurements of outdoor, indoor and in-transit samples over the selected two-week monitoring periods. We expect that some VOCs will have significant indoor sources.[48-51] Then, to refine and generalize our estimates to other locations within each city, we will use receptor modeling methods, including PMF and chemical mass balance, to derive independent estimates of traffic contributions to each VOC in outdoor, indoor and in-vehicle samples. This receptor modeling approach has proven successful in identifying the traffic-related VOC contributions in previous studies.[48-53] The indoor/outdoor ratios of these estimated traffic contributions to each compound will then be taken as the source-specific infiltration efficiency. Models of infiltration efficiencies will then be estimated across the entire MESA cohort in each city using residential survey information similar to the approach already developed for $PM_{2.5}$.[45]

<u>These individual-level predictions for total exposure will be employed in the epidemiologic analysis described in Objective 3. We will also assess the relative influence of each component (time spent outdoors, indoors and in-transit) on total exposure and the impact of using these different exposure metrics on health outcomes.</u>

Approach to Objective 3 - Epidemiologic Analysis.

This objective is the Primary Aim of Project 5. We propose to conduct a longitudinal assessment of the relation between individual-level exposure to traffic-derived air pollution and specific cardiovascular outcomes in the MESA Air cohort.

Study cohort and available data

The ongoing NIH/NHLBI-funded Multi-Ethnic Study of Atherosclerosis (MESA) includes 6,800 men and women, aged 45 to 84 years at recruitment with no clinically apparent cardiovascular disease at baseline. Participants were recruited between July 2000 and August 2002 (Exam 1) from six US locations: Baltimore County, MD; Chicago, IL; Forsyth County, NC; Alhambra, CA; Northern Manhattan and the Bronx, NY; and St. Paul, MN. This cohort was the primary recruitment source for MESA Air; 5,479 of these participants (93% of those approached) were recruited to MESA Air during MESA Exam 3 or 4. MESA Air also recruited an additional 490 participants from the MESA Family Study (PI, Jerome Rotter, Cedars-Sinai Medical Center), as well as 257 new participants from expanded areas around New York and Los Angeles to provide additional air pollution contrast. During the next clinic exam (Exam 5, scheduled for April 2010 through October 2011), all MESA participants not currently enrolled in MESA Air will be re-approached. Therefore, the current total number of MESA Air participants is 6,226, but this may increase by as many as 750 during the next exam. The current MESA Air cohort is balanced by gender (53% female), and exhibits the following ethnic distribution: 38% Caucasian, 27% African-American, 24% Hispanic, and 10% Chinese-American. Additional descriptive factors about this cohort can be found in Table 1. This is an ideal cohort for assessing incident cardiovascular disease.

Table 1. Descriptive statistics of the MESA Air cohort at baseline.

Category	**Frequency**		**Variable**	**Mean (SD)**
Age > 69 yrs	40%		Age	68 (10)
Current smoking	12.4%		BMI (kg/m^2)	28.6 (5.6)
Known hypertension	38%		Systolic BP	125 (20.6)
Known diabetes	11%		Diastolic BP	71 (10.2)
Known cancer	8%		Heart rate (60 sec)	62 (9.5)

MESA study participants have attended in-person clinic exams approximately every two years since study inception. All MESA Air participants recruited through either the main MESA study or from the new recruit locations are scheduled to attend MESA Exam 5. Each exam includes questionnaires, anthropometry, biologic specimen collection, and other components. The number of MESA Air subjects participating in various exam components is shown below.

Table 2. Number of MESA Air participants for whom relevant exam component results are available/anticipated, by exam. Note that this is a subset of all available health data.

		Exam 1	Exam 2	Exam 3	Exam 4	Exam 5
Questionnaires	Air Questionnaire			302	5729	6226
	Medical History	5476	5364	5331	6192	6226
	Residential History		5281		725	
Exam Components	Retinal Photography		5325			5736
	MRI- Cardiac/Arterial Wall/Tagging	4179	573		1144	5479
	MRI Carotid		913			
	Coronary Artery Calcium CT	5478	2558	2633	2081	3600
	Carotid US (IMT)	5426	2617	2753	1880	3600
	Lung Density (emphysema)	5478	2558	2633	2081	3600

For this proposed study, we will conduct a longitudinal analysis of progression of left ventricular mass index (LVMI) and change in retinal arteriolar diameter (as corrected retinal arteriolar equivalent or CRAE) in participants at each of the four study cities in which we are conducting

mobile monitoring (LA, Chicago, Winston-Salem, and Baltimore). LVMI was obtained in MESA Exam 1 (07/00 – 07/02) and will be measured again in Exam 5 (4/10 – 10/11); retinal arteriolar diameter was first obtained in MESA Exam 2 (07/02 – 01/04) and will also be repeated in Exam 5. Therefore, for LVMI, the period of progression is 10 years; for CRAE, progression will be measured over 8 years.

In both Exam 1 and upcoming Exam 5, LVMI, LV end-diastolic volume (EDV), and LV end-systolic volume (ESV) are obtained by cardiac MRI. Images are acquired by 1.5 Tesla MRI Scanners (SIGNA LX and CVi, GE Healthcare, Buckinghamshire, UK, and Somatom Vision and Sonata, Siemens Medical Solutions, Berlin, Germany), using a previously published protocol.[54] All MRI data are submitted to the MESA MRI Reading Center at Johns Hopkins Hospital for centralized processing using MASSsoftware, Version 4.2 (Medis, Leiden, Netherlands). LVMI is calculated using the DuBois formula for body surface area.

Retinal arteriolar diameter is measured via fundus photography in both Exam 2 and upcoming Exam 5. For each participant, the optic disc and macula of both eyes are photographed in a darkened room using a 45° digital nonmydriatic camera.[55] Trained graders blinded to all participant characteristics review all images at the University of Wisconsin in Madison using previously reported protocols.[56-58]

Genome-wide DNA Methylation analysis will be conducted as part of the NIH Roadmap Initiative grant to Yongmei Liu.(ES107650-01, "Epigenome-Wide Association Study of DNA Methylation and Atherosclerosis"), on specially prepared blood samples collected in all MESA participants at Exam 5. Through this project, in a staged approach (initial sample 800, remainder in replicate sample), she will assess methylation status in promoter regions of all 14,000 genes with CpG islands, in PBMCs, monocytes, and CD4+ T-lymphocytes.

Statistical Power

Based on the number of subjects in each city that will have LVMI and CRAE measurements (Table 3, below) available at two exams, we have estimated the power to detect effects of exposure to near-roadway pollution mixtures on longitudinal rates of change expressed as the difference between LVMI/CRAE at Exam 1 or 2 and Exam 5. We consider a binary exposure based on proximity within 50 meters of a major road, as we have previously done.[7] Approximately 20% of subjects in this project are hence in the "exposed" category. Of course, the spatially refined multi-pollutant model developed in Objectives 1 and 2 will provide a greatly enhanced ability to predict exposure to traffic-related pollution on a continuous basis, so the calculations are highly conservative.

For LVMI we assume a 1.5 $\mu g/m^3$ in the change from Exams 1 to 5 for the exposed group relative to the unexposed group--approximately one third of the observed effect of the homozygous variant of the ESR1 TA Repeat gene on LVM (in terms of % mean LVM/LVMI)[7,59] The variance of the change in LVMI is estimated based on cross-sectional data[7] and assumed (again, very conservative) correlation of ρ=0.8. Based on these assumption we will have 91% power to detect a longitudinal effect of traffic-related air pollution on LVMI (α=0.05).

A similar strategy for CRAE assumes an effect of 1.0 μm on the change between Exams 2 and 5 for the exposed group relative to the unexposed group. This effect size is consistent with the observed effect of aspirin in individuals using antihypertensive medication.[60] We estimate at least 83% power to detect a longitudinal effect of traffic-related air pollution on CRAE (α=0.05).

Table 3. Number of participants with previous and planned measurements of LVMI and CRAE.

	LV Mass		CRAE diameter	
	Prior exam	Exam 5	Prior exam	Exam 5
Baltimore	812	692	942	917
Chicago	867	794	1069	1054
Los Angeles	1049	919	1212	1300
Winston-Salem	704	635	973	968
Total	3432	3040	4196	4239

In addition to the novel longitudinal analyses of LVMI and CRAE planned for this study, we also propose to evaluate the performance of our refined exposure estimates for traffic-derived air pollutants from Objective 2B compared to the existing metrics developed in MESA Air for the primary health outcomes of that study. The primary MESA Air health outcomes are: 1) progression of subclinical cardiovascular disease, assessed by coronary artery calcification (CAC) and intima-media thickness (IMT); and 2) incidence of cardiovascular disease events, including myocardial infarction and disease mortality. MESA Air employs a spatio-temporal modeling strategy focused on ambient-origin $PM_{2.5}$; we will compare the inferential strength of a model employing those exposure estimates with one employing the traffic-derived multi-pollutant estimates developed here.

The approach to statistical analysis of the epigenomics data will be developed at a later date, and is anticipated to be a primarily exploratory analysis, unless preliminary data is available at that time that permits hypothesis-testing by assessing specific gene's promoter regions (such as FOXP3, as described). Our analysis will be developed in concert with Project 4. Dr. Liu's group at Wake Forest University will examine the association of epigenomic markers with extent of atherosclerosis, in a cross-sectional manner. As an initial approach, we will examine the anticipated small sub-set of genes that exhibit substantial variability in the extent of hypermethylation or hypomethylation, and examine independent relationships between these measures and long-term exposure to roadway-derived pollutants. In subsequent analysis we will take advantage of Dr. Liu's results and examine evidence that DNA methylation associations with atherosclerosis might be independently explained by our pollutant measures. Statistical approaches will be developed by the Biostatistics core, and include attention to issues of multiple comparisons, such as application of false-discovery thresholds.

Statistical analysis

For the continuous health outcomes (LVMI and CRAE) we will use multiple linear regression to estimate the health effects of exposure to each pollutant based on individual subject estimates of home exposure from Objectives 1A and 2B. The regression model for pollutant $q=1,\ldots,p$ will take the form

$$Y_{li} = \beta_0 + \hat{X}_{ls_iq}\beta_q + Z_{li}\beta_Z + b_l + \varepsilon_{li}$$

where Y_{li} is the change in LVMI/CRAE from Exam 1/2 to Exam 5, $\hat{X}_{ls_iq}$ is the predicted long-term annual average exposure at the home address of subject *i* in area *l* and Z_{li} is a vector of subject-specific covariates that are included in the model primarily to adjust for confounding. Several levels of covariate adjustment will be considered: Model 1 (crude model with minimal adjustment for age, sex, body size, ethnicity, and area-level random effects), Model 2 (adds adjustment for confounders not in the causal pathway), Model 3 (also includes additional confounders potentially in the causal pathway), and Model 4 (includes additional fixed effect

adjustment for site in order to obtain an average within-area effect of exposure). To strengthen the inferential power of our analyses, for each outcome we will specify the primary confounder model in an *a priori* analysis plan.

In order to further understand the roles of within-city and between-city variation in exposure we will consider an alternative formulation of the basic health effect regression model in which we partition the exposure into a citywide average $\bar{\hat{X}}_{ls_iq}$ and a within-city component $(\hat{X}_{ls_iq} - \bar{\hat{X}}_{ls_iq})$. The corresponding regression model is

$$Y_{li} = \beta_0 + \bar{\hat{X}}_{ls_iq}\beta_{qb} + (\hat{X}_{ls_iq} - \bar{\hat{X}}_{ls_iq})\beta_{qw} + Z_{li}\beta_Z + b_l + \varepsilon_{li}$$

where β_{qb} is the effect of between-city variation in exposure and β_{qw} is the effect of within-city variation. Even if $\beta_{qb} = \beta_{qw}$ (as would be expected if the pollutant being measured has the same properties across cities), the estimated values may be different due to measurement error or uncontrolled confounding. Comparing these two effect estimates will yield some insight into the dominant sources of bias (if any).

We will conduct diagnostics on the residuals from our regression models to determine if there is poor model fit or a need for additional terms to account for spatial structure in the outcome. Since we are using predicted exposures in place of the true values we need to account for measurement error that has the potential to introduce bias and to impact the standard errors, resulting in incorrect confidence intervals and inferential conclusions. A variety of techniques to correct for the effects of measurement error have been developed,[61] but the assumptions underlying standard methods do not hold when the exposure is predicted using the output of a spatial model as in Objective 1A. We will employ a version of the parametric bootstrap that we have recently shown to be an effective method for accounting for measurement error in this setting. [62]

In addition to analyses based on exposures at the subject homes, we will assess the impact of incorporating commuting patterns and time spent in traffic in the exposure predictions. We will use the multivariate regression model described above, except that we will replace $\hat{X}_{s_iq}$ with exposures derived from the prediction models developed in Objectives 1B and 2B to explicitly account for time spent in traffic.

To complement our estimates of health effects for individual pollutant components, we will employ a novel methodology in order to assess the risk associated with exposure to different mixtures of traffic-related pollutants. The objective of this novel analysis is to permit an analyst to specify two essentially arbitrary mixtures of pollutants and compare the relative effect of exposure to these two mixtures on the health outcome of interest. For example, we will be able to assess the difference in expected longitudinal changes in LVMI or CRAE associated with pollutant mixtures characteristic of different locations relative to roadways, traffic conditions, or atmospheric conditions. We will also be able to evaluate the effect of transitioning from a nominal pollutant mixture to a modified mixture resulting from implementing a new regulatory scheme or introducing advanced technology that differentially controls emissions of various pollutants. Details are provided in the Biostatistics Core (see the Biostatistics Core Research Plan for Objective 3a).

We will conduct a whole-genome epigenetic analysis using a vector of DNA methylation profiles as the outcome. The basic regression model for these epigenetic outcomes will be the

same as for the traditional health outcomes described above. The key difference is that, in light of the large number of outcomes being considered, we will account for multiple testing using the false discovery rate correction method (q=0.20),[63,64] and the results will be regarded as hypothesis generating.

Expected Results, Benefits and Outcomes

Project 5 of the UW CCAR integrates outputs from Project 1 to make the first application of a multi-pollutant approach to estimating exposure in an epidemiological study. As the first of its kind, it serves in some sense as a test case of the utility of a multi-pollutant approach in this context. Using the near-roadway exposure setting as a prototype of a multi-pollutant exposure somewhat simplifies the task of estimating population exposure to the entire complex ambient pollution mixture.

This project offers an innovative approach to estimating exposure to a mixture of pollutants. Using the detailed raw data generated by both the mobile and fixed site monitoring campaigns, exposure to contrasting aspects of the near-roadway pollution mix will be estimated. Following the ongoing approach in MESA Air to estimate individual exposure levels, contrasting near-roadway exposures will also be estimated at the individual level, in this case necessitated by the contrasts in concentrations and chemical and physical features of the mix that vary over very small spatial scales. The resulting exposure model will be the first of its kind to produce individual-level exposure estimates to a multi-pollutant mix. The use of personal monitoring and GPS locators in this study will allow us to determine the importance of on-road exposures and, equally importantly, the effect of ignoring this (with resulting measurement error) on estimates of health effect.

This epidemiologic study will allow estimates of effect of characteristics of near roadway exposure to be made on sub-clinical cardiovascular endpoints.. The sub-clinical endpoints chosen (left ventricular mass and retinal vascular changes) have been shown in MESA Air to be sensitive endpoints to individual-level exposure estimates of $PM_{2.5}$ concentrations and distance-based measures of traffic exposure. These endpoints would reasonably be expected to also be sensitive to the new near roadway exposure from the current study and thereby allow estimates of cardiovascular effect of near roadway exposures. Also unique is the attention paid to effects of exposure measurement error. Accounting for measurement error will be more valid than previous effect estimates that have not taken effects of measurement error into account.

EPA has expressed interest in a multi-pollutant approach to regulating air pollution, which requires focused novel approaches to research. In light of the central role that epidemiological findings play in the current criteria pollutant standard setting process, it is anticipated that epidemiological studies that take a multi-pollutant perspective will play a similarly critical role in the setting of multi-pollutant standards. This study will help set a precedent for how such studies are carried out and provide important insights for study designs and methods.

This research will also provide information on the role of in-transit, on-road exposures in contributing to overall estimates of exposure to near-roadway pollutants and their impact on estimates of health effect. These findings will determine whether it is important to collect on-road and in-transit data in epidemiological studies of near roadway exposures.

Identifying the features of the near roadway pollutant mix that are most strongly associated with our sub-clinical cardiovascular outcomes, when assessed in combination with our experimental findings from the toxicology and human clinical projects, will provide important information on

the role of background pollution and pollutant interactions in modifying the effects of traffic pollution on human health. This information will help focus policy relating to vehicle fuels and fuel mixtures and suggest policies that might vary by type of background pollution present. Control policies that focus on some of the more important characteristics of near roadway pollution identified in this study will improve the efficiency of air pollution policy and thereby potentially improve the ability to prevent the important cardiovascular effects of such exposure. The criteria pollutant approach to dealing with a single pollutant at a time does not allow insights into these issues.

General Project Information

A key component for this project is smooth integration with MESA and its ancillary study, MESA Air. Our research team is ideally situated to ensure seamless interactions in this epidemiologic study. Sverre Vedal, PI of this project, is also the PI of the HEI-funded NPACT study, another ancillary study to MESA Air, and is well-versed in the policies and procedures for data acquisition and sharing within MESA. Joel Kaufman, a co-investigator on this proposed project, is the PI of MESA Air and will ensure that this collaborative process is a smooth one. Both the MESA Steering and Ancillary Studies Committees have already approved this proposed study, and the PIs of the field centers at which we propose personal monitoring have executed subawards with us approving this work. In addition, Dr. Yongmei Liu, PI of the MESA Epigenetics study, Dr. Joao Lima, PI of the MESA MRI study, and Dr. Ron Klein, PI of the MESA Eye study, have all provided letters of intent to provide access to critical endpoint data for this project. MESA has a well-structured data sharing and publications protocol, which will ensure that we have access to all necessary data for our study.

Facilities and Personnel

The MESA Air laboratory provides the foundation for the field work component of this project. Our research scientists and technicians have developed and implemented air monitoring programs as part of MESA Air for the past five years, and are exceptionally prepared to lead the personal and residential air monitoring campaign described here. We will work with the staff of the UW Environmental Health Laboratory (EHL), whose staff includes PhD trained analytical chemists, to analyze all passive samples proposed in this study.

Project Schedule

As this project integrates information from other sources (Project 1 and the MESA/MESA Air studies, in particular), our planning must take into account the availability of necessary inputs. The expected timeline for this project is shown below in Table 4.

Table 4. Expected project timeline.

Activity	Year 1	Year 2	Year 3	Year 4	Year 5
Field prep, recruitment, consent (Aim 2A)	X				
Personal monitoring field work (Aim 2A)		X			
Sample analysis and data analysis (Aim 2A)		X	X		
Spatial model development from Project 1 (Aim 1)			X	X	
Development of individual-level estimates (Aim 2B)			X	X	
Integration of personal monitoring data (Aim 2A)				X	
Health analysis (Aim 3)				X	X

Quality Management Plan (QMP): UW Center for Clean Air Research (UW CCAR)

1. Summary. The University of Washington and the collaborating institutions in the UW CCAR are committed to producing quality environmental exposure and health data and analyses. As such, we will implement a quality assurance (QA) program for all monitoring, data gathering, sample and data transferring, laboratory analysis, data processing, and data analysis efforts. A key function of the QA program will be to document the methods used to collect and analyze data so that it will be scientifically valid and defensible. This QMP describes the elements of the quality system to be implemented by the UW CCAR and serves as the umbrella document under which individual quality activities are conducted. The QMP will be supported by the more specific Quality Assurance Project Plans (QAPPs) and standard operating procedures (SOPs).

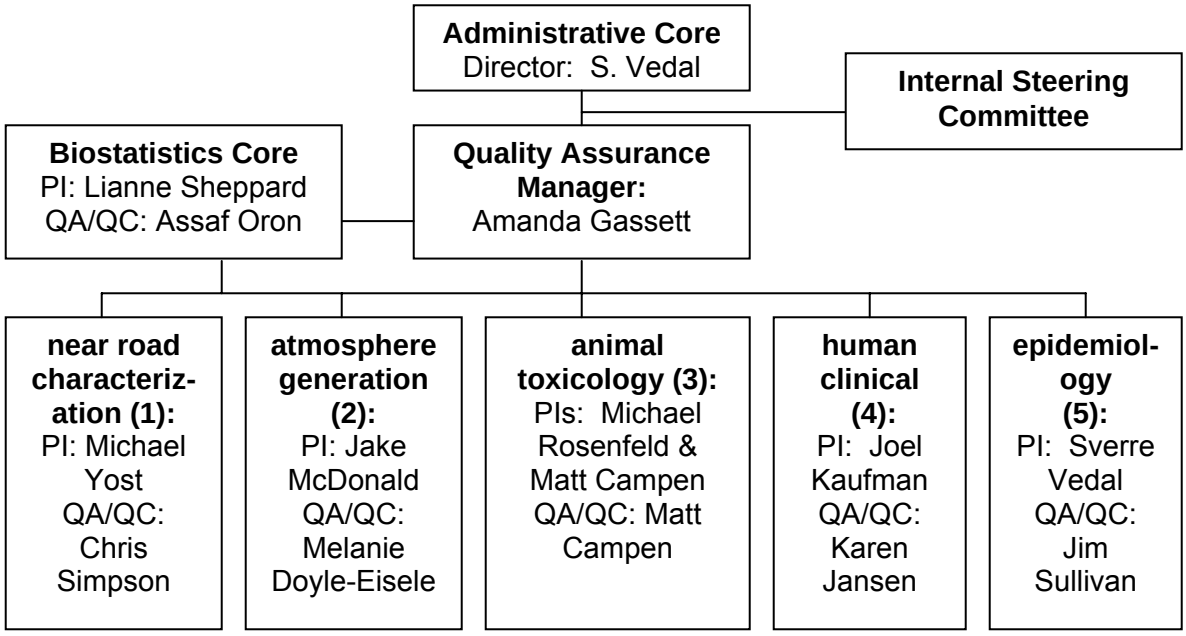
The UW CCAR is an integrated multi-project research program focused on the cardiovascular health effects of near-roadway pollution. Individual projects use and generate data and other outputs that differ to some degree from those of the other program components. This makes for a complex quality management process entailing a variety of QA/QC activities. Types of data include both secondary existing data obtained from many sources and primary data generated by our own air monitoring platforms and measurements. Besides strictly data, a program of QA/QC activities is also needed for monitoring, data gathering, sample and data transfer and storage, laboratory analysis, data processing and data analysis. The QA/QC needs are therefore varied and extensive.

This proposed Center either utilizes study procedures closely related to those of ongoing research projects and centers, or exploits linkages with them: at UW, most notably MESA (and its Coordinating Center [CC] in Seattle) and MESA Air, the Tacoma and Seattle Area Air Toxics Community Scale Monitoring Program, the monitoring and epidemiology portion of the National Particle Component Toxicity (NPACT) study, and the NIEHS DISCOVER Center; at LRRI, most notably the toxicology portion of NPACT, the National Environmental Respiratory Center (NERC) and the Advanced Collaborative Emissions Study (ACES). QA/QC protocols and other quality activities of these ongoing projects that already have well-developed and documented QA/QC activities will be exploited and integrated with the UW CCAR quality activities as appropriate.

2. Organization and Management. The organizational chart below identifies the components of the UW CCAR. The Center Director (Sverre Vedal) has ultimate responsibility for the quality aspects of the UW CCAR.

The Quality Assurance Manager (QAM, Amanda Gassett) is most directly responsible for the QA/QC activities of the Center. Amanda Gassett, BA (Computer Science/Math), will serve as the QAM for this Center. She currently serves as the Data Quality Officer for MESA Air, where her responsibilities include communication with field technicians, quality assessment of field data, review of lab data, resolution of quality issues in data, presentation of data quality to the MESA Air QA Working Group and Steering Committee. She also has experience authoring QAPPs, Data Organization and Operating Procedures (DOOPs) and technical SOPs.

The QAM acts as an independent authority organizationally removed from those who generate and use the Center's data and will oversee all quality aspects of the UW CCAC. The QAM has the authority to review all field and analytical procedures, data sets and analyses to verify quality of data generation, compilation and evaluation.



Because of the multiple projects of the Center, and with Project 2 and much of Project 3 taking place in Albuquerque at the Lovelace Respiratory Research Center (LRRI), and the partly dissimilar nature of the research activities that occur in the projects and core, the more immediate QA activities for each project/core will be the responsibility of each of the project/core QA/QC individuals identified above. These individuals will be responsible for producing project/core-specific QA/QC materials for review by the QAM. It is expected that the QAM will need to travel to Albuquerque once each year. The QAM will review the QA/QC activities and documentation of each component and will prepare an annual report of findings and recommendations to be presented to a meeting of the Internal Steering Committee (ISC, see Administrative Core description). Additional or more frequent reporting may be requested by the PI and the ISC as needed. The QAM will hold monthly QA/QC meetings at UW. Project and core QA/QC personnel are expected to attend these monthly meetings, and will include project investigators and staff as needed; QA/QC individuals at LRRI (M. Doyle-Eisele) and University of New Mexico (M. Campen) will join by videoconference. Karen Stukovsky of the MESA Coordinating Center (see Project 5 below) will also attend these meetings.

3. Quality System.

a. How research activities are reviewed and evaluated for quality.

The QMP for each project will include specific metrics of quality ("Data Quality Objectives") for all data collected. Each project will have a QA Subcommittee that will meet regularly to address any data quality issues that arise and to report on interim findings. The Biostatistics Core will meet regularly to review data and analysis issues

from all projects. As a matter of course, a process user or data collection technician will have initial responsibility for assessing the data derived from their process.

Each project will undergo an internal audit annually. This will include a review of the relevant quality documents, such as the QMP, SOPs and DOOPs. Each project will compile a summary of data collected, data quality objectives met, data quality issues to be addressed, and other progress made. An overall report compiling these summaries with recommendations will be circulated to the QA Subcommittees, the Biostatistics Core, and the principal investigators. These groups will approve, reject, modify, or add recommendations to the QA officers of each project. Each QA Subcommitee will oversee the implementation of any changes, with results audited by the larger oversight groups at an appropriate time. Changes may include changes to procedures and/or to QA/QC documents. The annual reports, reviews, and all versions of QA/QC documents will be archived on the Department of Environmental and Occupational Health (DEOHS) server. Any planned work of the UW CCAR using data may not begin until a QAPP has been written and approved for completeness by the QAM.

b. Staff training.

Training will be a key element of the quality system used for the UW CCAR projects. There will be training at two primary levels: (1) training of all involved personnel as to the purpose and content of the QMP; and (2) training of all technical personnel on all aspects of the SOP that they will be using. After SOP training, personnel will be evaluated first on their ability to complete procedures in the classroom setting, then later for their ability to complete procedures in the field. Recommendations for future training may be made based on the outcomes of the evaluations.

Study personnel will be certified as needed. Copies of certificates will be kept on file and reminders sent out near the time when or if the certification needs to be renewed. For non-certified jobs, performance-based standards will be developed. To start, training needs will be assessed based on anticipated requirements to do the job. As the study progresses, training needs will be re-assessed on a periodic basis. The need for training will be evaluated based on task performance and project needs. UW staff that will work with human participant data will complete UW's human subjects training, and each certificate of completion will be kept on file.

c. Data storage and availability.

Working versions of datasets will be housed in databases on UW DEOHS servers, or on local, secure resources maintained by LRRI or WSU. Final versions of all datasets that contain MESA health data will be stored on UW's servers at the Coordinating Center. Investigators that intend to publish results will receive data from the Data Manager. A copy of the data request and of the dataset will be archived and would be made available to any auditor upon request. Certain documents may be made available to UW CCAR collaborators through the internal UW CCAR website (subject to the approval of the PI).

d. Review, evaluation and change of QA/QC activities.

The QMP and QAPP will be reviewed annually along with the QA report on research activities. At that time, or at any time that a need arises for a change in the QA/QC procedures, the QAPP, QMP, or SOPs may be revised. Members of the QA

Subcommittee of each project are responsible for identifying areas of the QA/QC documents and procedures that need revision. Any revisions/new versions of QA/QC documents must be reviewed and approved by all of the appropriate investigators.

4. Project or Component Specific (abbreviated and selective).

Project 1. Exposure characterization. [Aim: characterize real-world near-roadway pollutant concentrations, particle size distributions and chemical composition.] QC measures (Data Quality Indicators [DQIs]) are established to evaluate and control various phases (sampling, preparation, and analysis) of the measurement process to ensure that total measurement uncertainty is within the range prescribed. QC procedures also include record keeping, use of monitoring blanks, and instrument calibrations and are used to maintain the measurement systems within prescribed limits of acceptability. QA procedures will be used assess whether these systems have achieved the desired DQIs. Data activities include recording, validation and verification, transformation and reduction, tracking, storage and retrieval and finally summarization and analysis. Data quality assessments will be performed to ensure that the levels of uncertainty meet the data quality defined objectives.

Project 2. Exposure generation (at LRRI). [Aim: simulate realistic contrasting near-roadway multi-pollutant exposure atmospheres for laboratory animal and human studies.] Once an exposure system has been developed, the conduct of the study includes: development of standard operating procedures (SOPs) for system operation and safety, pre-study exposure system validation; training and training certification of system operators; and conduct of exposures. SOPs document the conduct of all aspects of the study and the step-by-step procedures for operating the generation system, collecting environmental or exposure monitoring data, and safety. Institutional SOPs are reviewed and approved by LRRI management, and study-specific SOPs are approved by the Principal Investigator who oversees each function. Once an SOP is developed, each technician participating in that portion of the study is trained, and training is documented.

Project 3. Animal toxicology (at LRRI). [Aim: identify cardiovascular effects and pathogenic mechanisms of near-roadway exposures in animal models.] Most data will be collected by computerized measurement systems. Hard copies of the data are printed and stored in laboratory notebooks. Because we are proposing to develop or implement assays of new biological markers, we do not have specific SOPs in place. However, as we achieve success in demonstrating consistent and predictable responses that characterize the value of specific markers, we will optimize and protocolize assays to ensure consistency across studies. Power calculations are provided in the description for Project 3. Biological samples and all tissues are coded according to study protocol and date of collection, and all animal subjects are given unique identifiers. Calibrations are performed routinely on all systems used in the proposed work. Most biochemical and molecular assays depend on the generation of a standard curve. QC is implemented in the personnel training, standardization of protocols, and accuracy to the assay techniques. We further implement certain QA strategies by reviewing data for consistency and variance. Dr. Campen will audit data from the instrument through to statistical analysis.

Project 4. Human clinical study. [Aim: investigate vascular response to combustion-derived gases and particles in humans.] Specific QA procedures will be developed

related to exposure generation, intensive exposure characterization, session-specific exposure monitoring, processing of biological specimens, and physiological testing. SOPs for data gathering, data processing, data analysis, and quality control protocols will be developed. Technicians will be trained to ensure that the proper sampling and analysis procedures are followed. Established procedures for gathering, handling, processing and storing data and samples will be observed. A uniform coding and labeling system will be used for all specimens. SOPs will ensure that contamination is prevented and sample integrity is retained. All equipment and instruments will be calibrated according to fixed schedules and the analysis requirements of the SOPs. All data will be maintained in computer files with hard copies also maintained. All analyses will be documented.

Project 5. Epidemiology study. [Aim: identify effects of long-term exposure to traffic-derived particles and gases on sub-clinical measures of cardiovascular disease in a multi-ethnic cohort.] QAPPs that integrate all technical and quality aspects of this project including planning, implementation and assessment will be created for both the field monitoring data and the health outcome data and are in many instances already well developed. SOPs are also already in place for many of the activities to be carried out for this project. Because this project uses the MESA Air cohort, the MESA Coordinating Center (CC) in Seattle will play a major role in QA activities, especially those related to all health data, but also all data once they are linked with the health data. The MESA CC has day-to-day responsibility for all health data for MESA Air. Karen Stukovsky is the MESA CC staff project officer responsible for MESA QA/QC and support for her has been included in the Project 5 budget. MESA Air's approved QMP documents are located on the MESA Air intranet site and are available to the MESA CC.

Biostatistics Core. This Core will rely on existing MESA Air infrastructure for many operating procedures documented in the MESA Air DOOP. Included are data request procedures, methods for data evaluation, data storage and back-up, and instructions for use of the statistical analysis plan. This Core will develop models implemented with newly created software or by application of standard or commercially available software. We will document assumptions and user-specified options for all methods and analyses and summarize the accuracy or reliability of results and model sensitivity. Internal peer review of the results of this research, prior to data publication, will include review of the data quality checking, data management, and data analysis procedures and applicable programs by one or more of the research project investigators or staff.

5. Documentation and Records. The QMP is the overarching document that describes the system and how it will be implemented. This document will allow the QAM, study managers, and technicians to see how quality fits into the study and provides high-level direction for the operation of study systems. The SOPs detail study activities. The QAPPs will integrate all technical and quality aspects of the project, including planning, implementation, and assessment. The QAPP provides a project-specific "blueprint" to obtain the type and quality of data needed for a specific decision or use. The QAPP documents how QA and QC activities will assure that the type and quality of results are what is needed and expected. The QAPP will be completed prior to sample collection and be located on the UW CCAR document server for all UW CCAR staff to use. All versions of the QAPP, QMP, and annual QA reports will be archived.

Data Plan: University of Washington Center for Clean Air Research (UW CCAR)

Initially, all data, including primary data and existing (secondary) data, and other information from analyses or models developed, will be made available to the other investigators of the Center. Center investigators will have priority for publication of results employing these data. Center investigators will, however, be required to report findings using this information in a timely manner.

All data and other information used and generated by the UW CCAR will be ultimately made available to the scientific community. All data will be considered for data sharing. Data will be made as widely and freely available as possible while safeguarding the privacy of participants, and protecting confidential data. The information will be made available to the scientific community in a format and with documentation that will allow reasonable accessibility to the information without requiring specialized skills or familiarity with the data format so that other researchers can make ready use of these materials. Recognizing the public investment in the UW CCAR program, none of this information is considered proprietary. However, access and analysis of data in this repository will require completing an application process to be developed for the Center for either data analysis or for ancillary studies, as appropriate. All data can be made available to the EPA project officer without restriction, with appropriate confidentiality safeguards.

Because Project 5 data and other information is closely integrated with MESA (an NIH/NHLBI study) and MESA Air, much of the data collected under the proposed project will ultimately reside with the MESA Coordinating Center (CC) and be made available to other investigators, as is routinely the case within the parent MESA study. Within the established rules of the fully developed MESA data management system, the CC will subsequently make available all non-identifiable statistical data (including primary and secondary/existing data) from observations, analyses, or model development within this study in a format and with documentation such that other qualified researchers in the scientific community can use these materials. Although not considered proprietary, analysis of data in this repository also requires completing the application process for either data analysis or ancillary studies as appropriate. Release of data from the ongoing MESA contracts is subject to the established processes of the MESA project and NIH/NHLBI.

Proper documentation (metadata) will be produced to ensure that others can use the dataset and to prevent misuse, misinterpretation, and confusion. Metadata will provide information about the methodology and procedures used to collect the data, details about codes, definitions of variables, variable field locations, frequencies, etc.

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Administrative Core: University of Washington Center for Clean Air Research (UW CCAR)

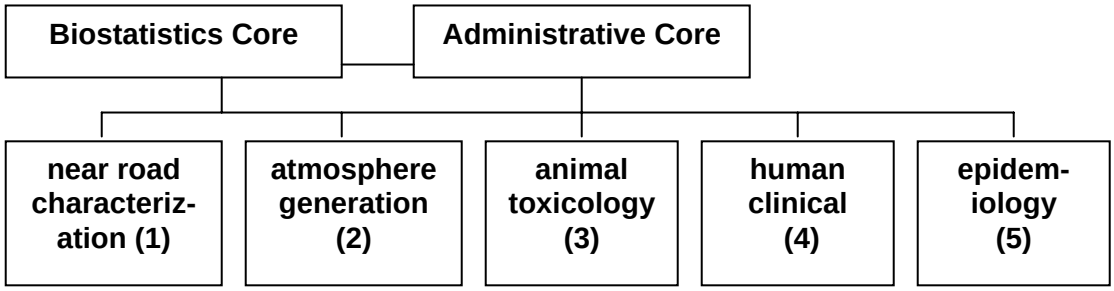
I. Description

The Administrative Core is responsible for the overall administration of the UW CCAR. The overall goals of the core are to: (1) ensure that the UW CCAR program of research functions effectively and efficiently using timely administrative and scientific review procedures; and (2) ensure that the UW CCAR investigators communicate effectively with each other and the broader scientific and regulatory communities, as well as with the other EPA clean air research centers and the EPA.

The proposed UW CCAR is a complex multi-disciplinary program that includes investigators spread across several departments at the University of Washington and three additional institutions, the Lovelace Respiratory Research Institute and University of New Mexico in Albuquerque, NM and Washington State University in Spokane, WA. While bringing this group of investigators together in this Center for a common purpose is a major strength, and we have demonstrated experience in assembling multi-disciplinary groups that work effectively together, a key to the success of this effort is a strong and effective Administrative Core.

II. Center Organization and Administrative Structure

The UW CCAR consists to two cores (this Administrative Core, and one facility support core, the Biostatistics Core) and five research projects (Exposure characterization, Exposure atmosphere generation, Toxicology, Human clinical, Epidemiology).



This organization was chosen to facilitate achieving the overall research goal of the Center and to maximize opportunities for scientific integration and efficient use of center resources. For example, the Biostatistics Core activities of developing and applying statistical methods could have formed an independent research project with integration across projects. However, it was elected to include these activities under core activities to facilitate a unified approach to the multivariate problems that cross project lines and to maintain a core of investigators working together on similar problems. Conversely, the Exposure generation project (Project 2) could have been organized as a core generating exposure atmospheres for the experimental health effects projects. However, the activities of the atmosphere exposure generation project were deemed to be sufficiently original to be best served as a project.

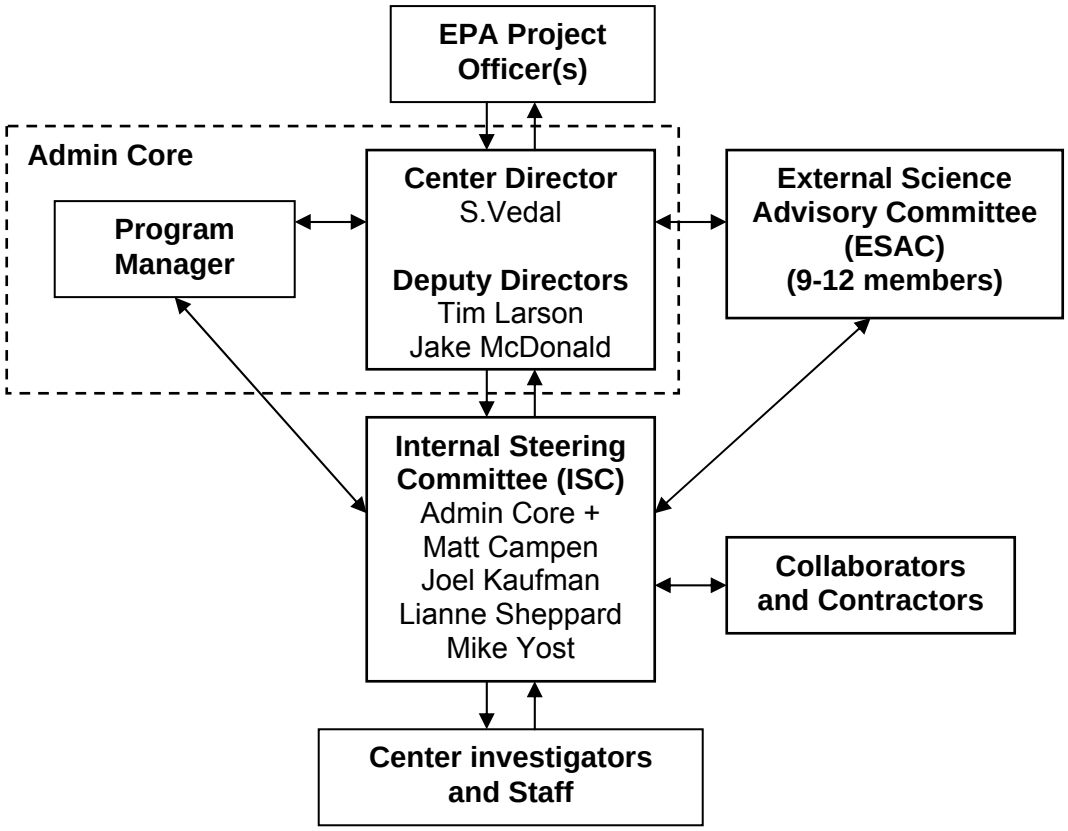
Center leadership. Leadership of the UW CCAR consists of a Center Director (Sverre Vedal) and two Deputy Directors (Tim Larson and Jake McDonald) who exhibit

complementary scientific expertise and interests thereby providing a broad perspective for the Center on the air pollution research field. One of the deputy directors (Jake McDonald) is at the Lovelace Respiratory Research Center (LRRI) and the other (Tim Larson) at UW. This facilitates both better communication and integration across the two main research sites of the Center, as well as more direct oversight at each site. The Center directors will directly supervise the activities of the Program Manager who will serve the administrative needs of all project and core principal investigators and all other Center researchers and staff; the Center Director, however, will have the most direct supervision of and interaction with the Program Manager.

Composition of the Administrative Core. The Core is made up of the Center Director, the two Deputy Directors and the Program Manager.

Center leadership is assisted by two committees, the Internal Steering Committee (ISC) and the External Scientific Advisory Committee (ESAC), who provide advice and oversight for the Center. The ISC consists of the Center Director and deputy directors and the principal investigators of the Biostatistics Core and of the other scientific projects who are not also Center directors.

The Center organizational chart below identifies components of the organizational structure and the avenues of communication:



III. Responsibilities of the Center Leadership

1. Center Director

The Center Director, Dr. Sverre Vedal, assumes overall responsibility for administration of the Center, overall scientific management, long-term planning and the ethical conduct of research. He will chair the Internal Steering Committee (ISC) and with the ISC facilitate integration of research projects to achieve maximum benefit from the multi-disciplinary structure of the Center. The ESAC will meet and provide overview, critiques and recommendations on research goals, progress and future directions just prior to the CCAR submitting its annual report to EPA. The Center Director will incorporate the ESAC's comments to formulate the annual report of research progress to the EPA Grant Administrators and ESAC. The Center Director will lead efforts to modify research plans as deemed necessary based on comments from both the ESAC and the EPA.

At least annually, the Center Director will request the Center Quality Assurance Manager to conduct an audit of the Center's quality system. The audit report will be submitted to the ISC for review, response, and action. All documentation will be provided to EPA.

The Center Director will coordinate research goals activities with the other EPA Clean Air Research Centers through monthly exchanges with the Centers Coordination Committee, participation in the annual Center Directors meeting, facilitation of research linkages between investigators at other Centers, and hosting at least one Centers research workshop during the five years of Center funding.

The Center Director will lead efforts to continue to seek additional funding to meet additional research needs or test new research hypotheses generated or suggested from emerging air pollution research findings, to educate stakeholders in areas related to multi-pollutant air pollution issues, to increase collaborative linkages with other UW and outside researchers, and to develop international awareness of current multi-pollutant research carried out by EPA Centers.

2. Deputy Directors

The Deputy Directors (Drs. Tim Larson and Jake McDonald) will work closely with the Center Director in overseeing the functioning of the UW CCAR. As part of the Administrative Core, the Deputy Directors will be actively involved in decision-making at all levels. As part of the ISC, the Deputy Directors will have close interaction with all UW CCAR investigators and help facilitate functioning of the ISC. In the event that the Center Director is unable to continue in the role of director, the Deputy Director at UW will assume that role.

The Center Director and Deputy Directors will together develop the following tools, guidelines, and criteria to facilitate research:

(i) A statement of role(s) of UW CCAR investigators and common research guidelines and procedures; (ii) Performance criteria approved by the ISC and ESAC to evaluate the effectiveness of research efforts; (iii) Milestones to evaluate the Center's overall progress in achieving its research theme; (iv) Mechanisms for adding or deleting investigators and for changing short-term research goals based on research findings and recommendations from the ESAC and EPA; (v) A plan for data sharing to ensure access for all Center investigators and collaborators to data and research resources generated under the

auspices of grant funding; (vi) A plan for listing co-investigators as authors on published manuscripts; (vii) A mechanism for non-PI Center researchers to meet regularly and share research efforts and findings, and to ensure multi-disciplinary linkages are strengthened and communications open; and (viii) A mechanism or plan to consider and respond to concerns of scientific communities.

3. Program Manager

The Program Manager, to be named, will be the day-to-day administrative conduit between the Center Director and Deputy Directors and the UW CCAP investigating team and staff. The Program Manager will be asked to provide information as requested to the ISC and will record minutes of the ISC meetings.

The Program Manager will manage the Center parent budget and all sub-budgets, will reconcile all budgets and provide monthly reports to all budget PIs, and maintain files of all original documents related to fiscal matters including contracts. The Program Manager will ensure timely submission of contract documents upon award.

The Program Manager will have active interactions with the EPA grant administrators in achieving the goals of the UW CCAP and meeting the interests of EPA. The Program Manager will provide assistance on compiling the Annual Report to EPA, and will maintain an EndNote publications file to be forwarded to EPA semiannually. All UW CCAR manuscripts will be maintained in a hard copy library and electronically on the Center server to facilitate access for all researchers, collaborators, and interested parties.

The Program Manager will receive Human Subjects IRB documents at the time they are submitted for internal review at the University of Washington, and transmit copies to EPA for simultaneous review. It is understood that no research project involving human subjects will proceed without EPA approval of IRB documents. The Program Manager will maintain files of all original IRB documents. The Program Manager will also monitor and maintain animal care approvals for all research involving animals.

The Program Manager will compile and edit a semi-annual Center newsletter detailing current research, collaborations, seminars hosted by the Center, and all other activities pertinent to the goal of communicating research findings to a broad group of universities, governmental agencies, for-profit and non-profit organizations, and private citizens. An electronic bulletin will be disseminated frequently to announce seminars, meetings, research defenses and dissertations, and news events pertaining to near roadway and other relevant air pollution issues. All media coverage and press releases will be maintained either in hard copy or added to the Center website and server.

The Program Manager will maintain the Center web pages on an ongoing basis. The web site will contain information about research projects, published manuscripts, workshops, and public meetings developed as the result of grant funding. Other than presentations at scientific meetings, the Center's web site may be the most effective mechanism to disseminate information about the multi-disciplinary research program to interested parties on an international level.

4. Internal Steering Committee (ISC)

The ISC's function is to provide scientific and administrative advice and coordination to the Center Director, and to assist the Center Director and Deputy Directors by providing

oversight and strategic planning for the Center as a whole and on all projects and cores, specifically. The ISC helps establish the scientific and technical direction of the Center, assists in the development of common research guidelines and procedures and the establishment of rules for access to resources of individual projects and cores, addresses intellectual property and authorship issues, and supports the Center Director and Deputy Directors in setting the leadership tone in the process of developing a cohesive research effort for the CCAR as a whole. The general operating strategy for the ISC will be consensus decision making.

A primary responsibility of the ISC is to bridge projects, core resources, and pilot projects for the purpose and gauging progress and efficiency, and to recommend changes of support and addition or deletion of activities as identified to maximize progress on the research goals of the large-scale multi-disciplinary Center theme.

Membership of the ISC consists of the Center Director, the Deputy Directors and the principal investigators of each project and core identified in the Center research plan. The ISC will meet at least quarterly, and more often as needed. The ISC will also meet annually with the ESAC ideally just prior to the Center's submission of its annual report of research progress to EPA, which coincides with the annual meeting of the ESAC with the UW CCAR leadership and investigators.

5. External Scientific Advisory Committee (ESAC)

The External Science Advisory Committee will provide scientific and administrative advice to individual project and core investigators and the ISC. It will be composed of at least nine but no more than twelve scientists not otherwise associated with the UW CCAR. The members will be appointed solely by the Center Director in consultation with the ISC and with the approval of EPA. The EPA grant administrator(s) will attend all ESAC meetings, but will not be member(s) of the ESAC.

The ESAC will meet once a year ideally just prior to the submission of the annual progress report to EPA. This ensures prompt consideration and inclusion of recommendations made by the ESAC about meeting research goals and making changes to the direction of future research. The ESAC will ask individual investigators to consider research issues, and will comment on the appropriateness of funding support to achieve project and core goals.

The Chair of the ESAC will provide a compilation report of comments submitted by all members of the ESAC within a time frame agreed upon at the annual meeting, taking into consideration the timing of the Center's annual report to EPA. The ESAC report will be posted on the Center's server, forwarded to EPA, and made available to all interested parties. The Center Director will provide a written response to the ESAC with help from the ISC and other CCAR investigators. This response will be posted on the Center's server, sent to EPA and the ESAC, and also made available to all interested parties.

IV. Center Internal Policies and Procedures

The Center administrative structure and the roles and responsibilities of the Center administrative leadership and committees were identified above. In the below the focus is on Center internal integration.

A. Use of Administrative and Facility Core Resources

All participating investigators will be encouraged to use the resources of the Administrative and Biostatistical Cores. Outreach efforts include weekly Journal Club/ Research Updates meetings, the regularly distributed e-bulletin, and regular project and core meetings that require the presence and involvement of the interdisciplinary team. The following detailing of Administrative Core activities and tasks provides an overview of the core tasks and resources.

The Administrative Core (the Center Director, the two Deputy Directors and the Program Manager) will support dissemination of research project information and results via a common server where all data, reports, and other information are stored as well as via newsletters, website, and electronic bulletins. The Administrative Core will maintain a common meeting calendar on the website as well as a contact roster of Center researchers. The website will provide links to recent news events, noteworthy air pollution information from outside resources, Center abstracts, research reports, and published manuscripts as well as slide presentations and project/core descriptions. The Administrative Core will be responsible for ensuring that a regular meeting schedule is maintained by the Internal Steering Committee, and will maintain comprehensive budget information for the Center so decisions about pilot projects or changes in research direction can be evaluated in a timely manner. The Administrative Core will be available to assist with or entirely manage the hiring of employees as well as performance evaluations and promotions. The Administrative Core will plan and execute all PM Center seminars and meetings with outside researchers. The Administrative Core will meet at least monthly to address Center tasks and issues of research progress, integration of research, communications, budgets, and coordination with outside collaborators and the other EPA Clean Air Centers.

The Biostatistics Core activities are described in detail in that facility core description. These include: (1) advise Center projects on data management and compilation; (2) ensure quality statistical design and analysis of Center research; (3) implement and develop statistical methods that are required for Center projects; (4) identify additional statistical methodological research that will advance Center projects and seek resources to perform such research; and (5) communicate and disseminate Center findings.
B. Central Integration Plan (CIP) for internal integration. Six aspects of CIP activities are detailed.

1. Programmatic and funding decisions.

The research direction of the Center is laid out in the research proposal in response to the RFA. During the course of the Center, it may be desirable to make changes to the direction of research based on, for example, interim project results, new scientific information or hypotheses or disappointing project productivity. Similar considerations would result in modifications and reallocation of budgets.

a. Programmatic decisions. Suggestions for changes in the program of research that do not alter the original scope of work are welcome. These suggestions would typically come from Center investigators either as members of the ISC or individually, or from the ESAC. Final decisions on changes to the program of research will be made by the ISC after thorough discussion at the regular ISC meetings. Substantial Changes in the program of research will need the approval of the EPA Project Officer.

b. Funding decisions. No researcher will be allocated funds without having an adequate research protocol and budget documentation that has been provided to the Center Director and the ISC for review and approval at a set time each year as determined by the ISC. Allocation of grant funds will ultimately be determined by the ISC.

c. Adding/dropping participating investigators. An internal mechanism for adding investigators, or dropping investigators that have not been productive, will be developed by the Administrative Core and approved by the ISC within six months of the grant award.

2. Achieving timely research objectives.

Oversight of the pace of research will be the direct responsibility of the ISC with assessment and advice from the ESAC. Adherence to proposed project timelines will be assessed at the regular ISC meetings and will be based on presentations of research results at the regular Center research seminars and the annual Center meetings. Failure to adhere reasonably to the proposed timelines will need to be well justified to avoid being considered susceptible to reallocation or pulling of project funding to allow higher levels of support for more productive or higher priority projects.

3. Investigator communication.

While communication between investigators working in a center such as this might be expected to be commonplace, various demands on investigators' time and conflicting schedules can place roadblocks to communication. Roadblocks to effective communication are even greater between collaborating groups in a center that are separated geographically and administratively, such as the groups in this Center at the University of Washington, Washington State University (WSU), University of New Mexico (UNM) and Lovelace Respiratory Research Institute (LRRI). Several mechanisms are in place to keep communication between investigators in an institution, and between investigators from the collaborating institutions, active and constructive.

a. Seminars. First, Center investigators are expected to regularly attend and participate in the scheduled Center seminars. These include the biweekly Center meeting in which a Journal Club will alternate with a Research Seminar. This meeting will typically take place at the University of Washington, with investigators from WSU, UNM and LRRI attending by telephone or videoconferencing. In the Journal Club, individual investigators and other participating members of the research team will select one or two recent published papers for critical review. For the Research Seminar, individual investigators from each project will present an update on progress and findings; two presentations, one each from a different project or core will be planned.

b. ISC meetings. Members of the ISC, as PIs of their respective projects and cores, will communicate results of substantive discussions in ISC meetings to the other investigators and staff of their respective Center components.

c. Sharing of Research Resources. Recognizing that the nature of a Center grant is to move an entire field forward, the Center will ensure all investigators have equal access to research resources generated by collaborative research projects funded by the award. It is understood that data sharing is required, and dissemination of data and information by Center investigators is critical to the fundamental purpose of a Center grant. The Center

Director and Deputy Directors take the lead in showing a genuine commitment to share research resources among all the projects and cores. Resources to be shared include data and information, materials such as mutant animals, novel laboratory techniques, and computational tools developed for analysis, visualization, or data modeling.

d. Publications. A plan for listing co-investigators as authors on published manuscripts is required to ensure all co-investigators are recognized for contributions to individual research projects. This plan will be developed in the first year of funding, and will be called upon whenever manuscripts are submitted for publication. The plan will stipulate an internal review mechanism ensuring all co-investigators are fully aware of manuscript development and are able at minimum to participate in the review process.

e. Research Guidelines. A clear mechanism for implementing common research procedures was developed under the previous Northwest PM Center through the Statistical Analysis Plan developed by the Statistics and Data Core at that time. That mechanism will be exploited here and modified to be more relevant to the current Center. Investigators will be encouraged to provide feedback and criticism on all research plans from inception of research concepts through manuscript submission and review.

4. Monitoring and measuring progress in outputs/outcome.

a. Milestones and Evaluations. Milestones in achieving the Center's research theme upon which progress will be evaluated annually will be determined by the Internal Steering Committee (ISC) within six months of funding. Progress will be assessed and milestones updated annually at the award anniversary dates, will reflect recommendations of the ESAC, and will be incorporated into the annual progress report to EPA.

b. Performance Criteria. Performance is evaluated through successful data collection, progress, data sharing, and analysis as well as manuscript publication. Additional criteria to evaluate performance will be considered as suggested by Center investigators, and will be included in the ISC meeting agendas.

c. Progress in Outputs/Outcomes. Outputs of this Center include the research findings presented in abstracts and peer-reviewed publications, the new methodologies developed, including those relating to generation of exposure atmospheres and estimation of individual-level exposures to a pollutant mixture, and computational tools including annotated source code. Progress will be gauged from materials presented by the individual projects and core to the ISC and evaluation by both the ESAC and the ISC. The ESAC will play an important role in determining whether Center activities are having or likely to produce the proposed outcomes.

5. Responsibility for setting program priorities.

a. Short-Term Goals. The primary recognition of changes in short-term goals necessitated by research findings rests with individual investigators. It will be their responsibility to inform the ISC and co-investigators in a timely manner to facilitate the reallocation of resources within the Center to meet immediate needs that are accepted by the ISC as valid. On a yearly basis primary weight for making changes in short-term goals will be provided as internal recommendations to the ESAC for its consideration and response. Recommendations made by EPA and the ESAC will guide the Center ISC in making final decisions to change short-term goals.

b. Program Priorities. Original program priorities are proposed in the research proposal in response to the RFA. Change in overall program priorities will require combined consideration by EPA, the ESAC and the ISC.

6. Administrative responsibility for CIP implementation and compliance and evaluation of impact of the CIP in center integration.

Implementation of the CIP will be the responsibility of the Administrative Core, specifically the Center Director, the two Deputy Directors and the Program Manager. The Program Manager will be responsible collecting information relevant to CIP activities for the purposes of assessing compliance with the CIP and for evaluation of the impact of the CIP in integration Center activities. Evaluation of CIP impact be included on the meeting agenda of the ISC.

V. Center External Policies and Procedures

A. Communication with EPA

Communication with EPA will occur largely through members of the Administrative Core (the Center Director, the two Deputy Directors and the Program Manager). The Administrative Core will cover trip expenses for Center investigators to attend the annual EPA Centers meetings and the EPA Clean Air workshops. The Core will also host one of these Centers meetings and one of the workshops in Seattle.

Human Subjects. Prior to implementing human subjects recruitment and concurrent with submitting documents for internal UW review, the UW CCAR will submit the same documents to EPA for review and approval. All original documents (including yearly status reports and modifications) will be maintained by the Administrative Core for immediate submission to EPA. All RFA documents approved by UW Office of Sponsored Programs (OSP) are forwarded to UW Human Subjects Division for notification of pending research funding.

Animal Welfare. All RFA documents approved by UW OSP are reviewed and approved by the UW Institutional Animal Care and Use Committee (IACUC) and will be submitted to EPA. All animal research protocols are carefully reviewed on an annual basis. All researchers involved with animal studies in this RFA at UW have current UW IACUC approvals.

B. Integration among centers

EPA requirements include several formal mechanisms for integration among centers, in addition to informal interactions between centers and investigators that will take place. These formal mechanisms include: annual Centers meeting to review research progress, with one hosted by UW during the duration of the award; annual Centers workshops with one hosted by UW; individual Center reserved budgets to support studies with other Centers that are of a comparative or collaborative nature. These activities have been

budgeted in the Administrative Core budget and are included in the Administrative Core budget and budget justification.

C. External Dissemination and Communication

Research findings from the UW CCAR will be published in a timely manner in the peer-reviewed scientific literature.

The Administrative Core will take the lead in educational activities, information sharing, and outreach in the area of multi-pollutant air pollution research. With the Center Director's and Deputy Directors' guidance, the Program Manager will on a continuing basis work to enhance the value of the research, to educate stakeholders in areas related to near roadway air pollution, by increasing collaborative linkages with other UW and outside researchers, and by developing international awareness of current multi-pollutant research carried out by EPA Centers.

The Program Manager will compile and edit a semi-annual Center newsletter detailing current research, collaborations, seminars hosted by the Center, and all other activities pertinent to the goal of communicating research findings to a broad group of universities, governmental agencies, for-profit and non-profit organizations, and private citizens. An electronic bulletin will be disseminated frequently to announce seminars, meetings, research defenses and dissertations, and news events pertaining to near roadway and other relevant air pollution issues. All media coverage and press releases will be maintained either in hard copy or added to the Center website and server.
The Program Manager will maintain the Center web pages on an ongoing basis. The web site will contain information about research projects, published manuscripts, workshops, and public meetings developed as the result of grant funding. Other than presentations at scientific meetings, it is anticipated that the Center's web site may be the most effective mechanism to disseminate information about the multi-disciplinary research program to interested parties nationally and internationally.

Biostatistics Core

1. Objectives

The overall objective of this Core is to support the database management and statistical needs of all Center activities. This will be achieved through the following specific objectives:

1. Advise Center projects on data management and compilation
2. Ensure quality statistical design and analysis of Center research
3. Implement novel statistical methods that are required for Center projects: Develop an analytical framework for quantifying the health effects of different mixtures of air pollution components in a cohort study (Project 1 and Project 5)
4. Identify additional statistical methodological research that will advance Center projects
5. Communicate and disseminate Center findings

Rationale

Biostatistics support of air pollution research requires increasingly skilled personnel and specialized methods. Datasets and scientific questions have become increasingly complex and new methodology in statistics and biostatistics is currently developing very rapidly. It is more difficult than ever for air pollution science experts to acquire the full range of statistical technical skills to plan and carry out sophisticated data analyses. Even the most statistically savvy life science investigator finds it difficult, if not impossible, to keep up with advances in statistics and biostatistics. High quality data and analysis contributes to the overall potential for high quality, statistically sound research. The planned Biostatistics Core provides (bio)statistical support that enables Center investigators to incorporate rigorous statistical analyses and advanced statistical methods into their research. This Core will be a powerful unifying force in the UW Center for Clean Air Research by engaging in interdisciplinary interactions, supporting multiple projects, and ensuring careful attention to proper application of statistical methods. It will provide the crucial link between content area and statistical methodology experts that fosters the most scientifically relevant methodological development. Thorough and continuous involvement of statisticians from the design phase through the data analysis and interpretation phases of studies will be key to the strength, quality, impact, and recognition of the Center research.

2. Approach/Activities

The Biostatistics Core will serve every Center project through its services and activities. These are aligned with the objectives.

Objective 1: Advise Center projects on data management and compilation

While the project investigators are responsible for their data, this Core will support Center data management and compilation by providing advice on the following activities:

i. *Database design*: Data for each project will reside in one or more databases. Each database will be designed to ensure the most economical storage, limit redundancy, and ensure the data can be linked for analysis. Large projects with multiple data streams will require careful database design to ensure these features are present.

ii. *Forms design*: For forms that will undergo data entry, this Core will be available to consult with project investigators to improve the design and facilitate the data entry process. Eventual data analysis is an important consideration in the forms and database design process.

iii. *Data entry support*: Members of this Core will be available to advise on the design of computer data entry forms to transfer data from forms to database files in order to support the functionality of the data entry forms and the integrity of the data entry process. Double data entry with follow-up verification of any data that aren't identical in both entries is part of the data entry process.

iv. *Data quality review*: In collaboration with the project investigators, this Core will advise on procedures to routine validation checks to detect out of range or illegal values. Resolution of errors and inconsistencies as identified through the routine validation checking procedures is a project function. Included in data quality review is a system of flags to mark data that are questionable for some reason (e.g. below the LOD) and are stored in the database.

v. *Data storage and back-up*: Funds have been requested to allow Center data to be stored centrally. Central storage ensures the data are a) available for multiple uses and investigators, b) stored systematically, c) documented, and d) routinely backed up. Each project investigator will determine the stage of the data collection and editing process when it is most appropriate to include his/her data in the central storage system. The stage will depend on data collection methods and the extent of quality control activities that are required.

vi. *Documentation*: All procedures and databases, including variables, values, valid ranges, etc. will be documented. Members of this Core will be available to advise on data documentation.

Due to resource limitations, the role of this Core in database management and compilation will be advisory. Established MESA Air and other ancillary study infrastructure will provide much of the needed support for these activities.

Objective 2: Ensure quality statistical design and analysis of Center research

Objective 2a: General activities

This is one of the most important Core activities. The faculty and staff of this Core meet regularly (weekly) and these meetings are a forum for ensuring regular attention to the vast array of (bio)statistical needs of the Center. This Core will support these needs through the following general design and analysis activities:

i. *Study design*: The quality of many scientific studies can be improved by active dialogue with statisticians at the design phase. As Center needs evolve, the faculty and staff of this Core will consult with project investigators on the design of new studies.

ii. *Sample size calculations*: For many studies sample size calculations require more than the standard two-sample t-tests that have readily available formulas. For large or complex studies, this Core will enable sample size calculations using more sophisticated approaches such as simulation.

iii. *Data collection*: Input on data collection procedures and documentation of these procedures can improve the quality of the eventual analysis. As requested by project

investigators, this Core will provide the statistical perspective to data collection planning, documentation, and ongoing monitoring of data collection.

iv. *Statistical analysis plan (SAP) development*: The statistical analysis plan (SAP) procedure[1] emphasizes discipline and documentation of statistical analyses. The SAP summarizes the goals of the work, the hypotheses to be tested, translates the hypotheses into specific models with testable parameters, describes the analysis approach, lists important variables (outcome, predictor, and confounding), and documents the steps taken as the analysis unfolds. This process streamlines the analysis process by clarifying the goals and procedures, and facilitates the interpretation of study results by distinguishing analyses in the spectrum of hypothesis testing to hypothesis-generating objectives. Adherence to the SAP process improves statistical practice by ensuring a priori hypotheses are specified and approaches (to e.g. model selection, confounder selection and adjustment) are clarified in advance. Pre-specifying analyses in an SAP reduces reporting of inflated research results, which is prone to occur when analyses are selected after the data are available. This is an important problem in air pollution and health research because of the typically small effects of air pollutant exposures. The SAP will be an important tool in the Biostatistics Core's efforts to encourage sound statistical practice. Each proposed analysis (e.g. for a paper) will be pre-specified in a SAP. See the Figure at the end of this Core proposal for the SAP template.

v. *Statistical analysis*: Analyses will be conducted by many Center investigators and staff, including members of the Biostatistics Core. Oversight of analyses will be provided by Core faculty through the regular Core research meetings and in other forums. All analyses will have a SAP.

vi. *Interpretation of study results*: Subtle features of data, models, and analysis approaches can affect the interpretation of results. This Core will provide the statistical perspective and expertise on results interpretation.

Objective 2b: Brief overview of data analysis activities by project

This section gives a brief summary of the types of data analysis activities this Core expects to support for each project. More details are available in many individual project descriptions.

Project 1 is a field exposure study. One of its primary objectives is to characterize spatial and temporal variation of a variety of exposure measures with the ultimate goal of improving exposure predictions. Multivariate continuous data will be measured at a fine spatial and temporal scale. Novel statistical methods for these data are described in the next section; we will also conduct a thorough evaluation of the data, including assessment of the sensitivity of the prediction models derived from these data to many features of the data and analysis approach. In particular, we will evaluate the sensitivity of results to the amount of temporal and spatial averaging, the data collection design and richness of the data, and the prediction model assumptions.

Project 2 is an experimental exposure study with a primary focus on developing atmospheres to be used in toxicology and clinical studies. Data for each atmosphere will be in the form of a multivariate vector of continuous measurements at equally spaced time intervals. We will support the characterization and comparison of these atmospheres through statistical analysis

activities such as summarization and display of data, assessment of their stability over time and with replication, and testing of hypotheses to compare newly generated atmospheres to benchmark emissions.

Project 3 uses experimental toxicology research to test pre-specified hypotheses regarding vascular and immune system responses. Responses are typically expressed as continuous measures from individual animals. The Biostatistics Core will work with project investigators to refine the design of individual studies, conduct or assist with statistical analyses, including development of analysis plans and careful evaluation of model assumptions (e.g. the normality assumption for ANOVA), and interpret study results.

Project 4 is a clinical research project using a crossover design. While the final study design awaits early results from Projects 2 and 3, this Core assisted with the initial design and will support its planned refinement. The primary response data are expected to be continuous measures of vascular response. Once data have been collected, this Core will assist Project 4 with data analyses, assessment of the sensitivity of results to assumptions (such as the assumption of no carryover effect), and interpretation of study results.

Project 5 is observational and relies on epidemiological cohort study data. The Biostatistics Core expects to have extensive involvement with this project because of its reliance on methods for predicting and estimating health effects for multivariate mixtures to be developed by Core statisticians (see next section for details). In addition, all of this project's aims will require detailed data analysis to evaluate the quality of the spatial concentration prediction models (Objective 1), incorporate additional information into exposure prediction models (Objective 2), and assess the impact of these exposures on health outcomes, with appropriate correction for the measurement error induced by predictions from spatially misaligned data (Objective 3).

Objective 3: Implement novel statistical methods that are required for Center projects

Objective 3a: Quantifying the health effects of multi-pollutant mixtures in a cohort study

The Core will develop a set of tools that allow an analyst to specify a baseline multi-pollutant mixture of components and to calculate relative risks/hazard ratios for alternative multi-pollutant mixtures. These methods will be incorporated into Project 1, Objective 2 (mobile monitoring) and Project 5, Objectives 1 and 3 (epidemiological analysis).

3a.1. Background and Approach

Consider a cohort study with N subjects and suppose that each subject $i=1,\ldots,N$ at location s_i has a p-dimensional multivariate exposure vector X_{s_iq} ($q=1,\ldots,p$) and health outcome Y_i. The exposure vector is the concentrations of a p-dimensional mixture of air pollution components. We are focusing on chronic health effects, so X_{s_iq} represents a long-term (annual) average exposure for each pollutant. The health outcome of interest could be continuous (cross-sectional or longitudinal) or it could be a survival time. We will primarily describe our methodology for longitudinal outcomes expressed as a difference between an observed continuous outcome at two

follow-up exams, since these are the main outcomes considered in Project 5, Objective 3. We will note where modification is necessary for more general longitudinal or survival outcomes.

One approach to assessing the association between $X_{s_i q}$ and Y_i is to conduct separate analyses for each component of $X_{s_i q}$. We will conduct such analyses, but we are also interested in assessing the association between Y_i and multi-pollutant mixtures. There are three challenges in performing such an assessment that we will address with new methodology:

i. The multivariate exposure $X_{s_i q}$ cannot be measured directly for each of the subjects and needs to be estimated based on environmental monitoring data using a model that accounts for correlation in space and between components.
ii. Since the *p* components in the exposure mixture are likely to be correlated and to interact with each other in their associations with the health outcome, it is difficult to parameterize and interpret the coefficients from a multi-pollutant disease model.
iii. Estimated exposures are subject to measurement error that needs to be accounted for to ensure correct inference from the health effect analysis. Since different components of the exposure mixture may be subject to different degrees of measurement error, there is the possibility of confounding between components.

We propose an integrated methodology that addresses these challenges. We will construct a multivariate exposure model to estimate the concentration surface $\hat{X}_{s_i q}$, accounting for correlations in space and between pollutants. To facilitate fitting and interpreting the disease model, we will reduce the dimension of the exposure by principal component analysis, giving the principal component surface $\hat{X}_{s_i q'}$ ($q' = 1, \ldots, p', p' \leq p$). We will apply recently developed bootstrap measurement error correction methods in the health analysis to account for uncertainty in predicting the principal component exposure surface $\hat{X}_{s_i q'}$. Finally, we will make inference about the health effects of arbitrary *p*-dimensional pollutant mixtures, based on approximation in the p'-dimensional space spanned by the principal components.

3a.2. Previous Work

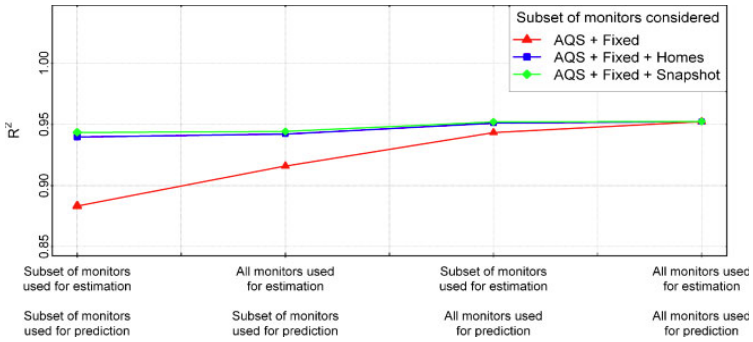
In order to estimate the health effects of air pollution in a cohort study, it is useful to exploit spatial heterogeneity in air pollution concentrations within a geographic region to assign exposures to individual cohort members. Since it is typically not feasible to measure the exposure of interest at each subject's location, we employ spatial or spatio-temporal statistical methods to predict exposures based on monitoring data.

As part of the MESA Air project, Drs. Sampson, Szpiro, and Sheppard have developed a spatio-temporal model to predict concentrations of a single air pollution component based on monitoring data that includes the EPA Air Quality System (AQS) and additional project-specific monitoring.[2,3] The form of the model is as follows

$$X_{st} = \mu_{st} + \nu_{st}$$
$$\mu_{st} = \beta_{0s} + \sum_{l=1,\ldots,k} \beta_{ls} f_l(t), \quad \beta_{l\cdot} \sim N\left(S_{l\cdot}\alpha_l, \Sigma(\theta_l)\right) \ (l = 0,\ldots,k) \qquad (1)$$
$$\nu_{\cdot t} \sim N\left(0, \Sigma_\nu(\theta_\nu)\right) \ \ (each\ t)$$

This hierarchical structure accounts for complex spatio-temporal dependencies by modeling spatially varying temporal trends as a linear combination of the mean-zero empirical seasonal basis functions $f_l(t)$.[4] Our modeling is done at the two-week time scale, which is consistent with the averaging period of our monitoring data. The spatial field of coefficients for each temporal trend is described by a universal kriging model that incorporates dependence on geographic covariates S_{ls} and spatial correlation in the residuals described by a covariance function $\Sigma(\theta_l)$, with the parameter θ_l incorporating the range and sill for a pre-specified geostatistical variogram model. The spatio-temporal residual field is modeled as spatially correlated but temporally independent because the seasonal trend basis functions account for autocorrelation in the two-week average data. The spatial covariance model for the residual field is $\Sigma_\nu(\theta_\nu)$, with the parameter θ_ν including the range, sill, and nugget for a geostatistical variogram model. This hierarchical model can exploit temporally sparse monitoring data with different sampling times at a large number of locations to improve predictions. The benefits of incorporating temporally sparse data are illustrated in Figure 1. Efficient computation is possible with maximum likelihood methods.[2] Due to the nature and complexity of the monitoring data being collected for this Center (see the proposal for mobile monitoring in Project 1, Objective 2), our primary prediction model will be spatial (with adjustment for time in a pre-processing step). A spatio-temporal model similar to the one described above will be used to allow for a more flexible temporal adjustment if this is warranted.

Figure 1: Simulation results showing the benefit of temporally sparse measurements for predicting long-term averages. AQS and Fixed sites are temporally rich, Homes and Snapshots are temporally sparse with irregular sampling patterns.



Air pollution cohort studies conducted by our research group and others have focused primarily on single air pollutant exposures.[5,6,7] There is a growing recognition in the literature that it is important to evaluate the effects of multi-pollutant mixtures, but little work has been done to address challenges such as correlation between pollutants, multivariate measurement error, and effect modification.[8] Some two-pollutant time-series studies have addressed inter-pollutant correlation and measurement error,[9,10] but the methods that have been used have limited applicability to higher dimensional exposures or to cohort studies.

Measurement error from using predicted exposures in place of the true values can affect inference for health effects by introducing bias and by inflating the standard errors.[11,12] A variety of techniques to correct for the effects of measurement error have been developed,[13] but the assumptions underlying standard methods do not hold when the exposure is predicted using a

spatio-temporal model such as equation (1). Recent work by Drs. Szpiro and Sheppard clarifies the impact of measurement error when the critical spatial heterogeneity in exposure is predicted using spatial or spatio-temporal statistical methods.[14]

The standard measurement error categories of classical and Berkson error do not apply exactly, largely because the errors are correlated and heteroscedastic. But it is useful to decompose the measurement error in the spatial setting into a classical-like component and a Berkson-like component. Like Berkson error, the Berkson-like component does not introduce bias in a linear health model but does inflate the standard errors.[14,12] In a nonlinear model, the Berkson-like component can be expected to induce limited bias when relative risks are small.[13] Like classical measurement error, the classical-like component has the potential to introduce bias and to alter the standard errors for effect estimation.[14]

We have shown that a version of the parametric bootstrap is an effective method for accounting for measurement error that results from using predicted exposures based on a spatial or spatio-temporal model.[14] See also Madsen et al.[15] The strategy is to estimate the underlying data-generating mechanism based on the available data and then simulate multiple bootstrap samples from this data-generating mechanism in order to derive empirical estimates of bias and standard errors for health effect estimation. We will extend the parametric bootstrap to the multi-pollutant exposure setting.

3a.3. Research Plan

The first step in our proposed multi-pollutant analysis is to predict a multivariate concentration field, allowing for correlations across space and between components; see the proposals for Project 1, Objective 2 (mobile monitoring) and Project 5, Objective 1 (epidemiological analysis) for additional background and motivation.

We will implement a universal co-kriging model with a land-use regression (LUR) structure for the mean. The data for our universal co-kriging model will come from the mobile monitoring campaign proposed in Project 1, Objective 2. Because the mobile monitoring data are collected from a moving platform during two sampling periods over the course of a single year, we need to adjust for temporal trends before including the measurements in a spatial prediction model. Our approach to dealing with short-term and long-term seasonal trends is described in the proposal for Project 5, Objective 1. Where possible, we will adjust for temporal trends using a single fixed site monitor that collects data during the mobile monitoring sampling period. If there is evidence of spatial variation in temporal trends (at either the short-term or long-term time scale), we will exploit additional data from regulatory monitors and employ a spatio-temporal model similar to the one developed for MESA Air (equation (1) above) as a basis for a more refined adjustment approach.

The covariates for each pollutant will be selected using a method similar to one recently developed for standard LUR models.[16] We will assume

$$X_{.q} = S_{.q}\alpha_q + \eta_{.q} \qquad (2)$$

for each component ($q=1,\ldots,p$), with correlated residuals between pollutants that have different marginal spatial covariances. The correlation of residuals will be based on co-kriging with a

"linear model of co-regionalization" (LMR) covariance construction (Banerjee et al. 2004). For the LMR construction, we start with *p* independent mean-zero spatial fields ω_{sq} with different correlation structures

$$\omega_{.q} \sim N\left(0, \Sigma(\phi_q)\right) \ (q = 1, \ldots, p) \tag{3}$$

and then define the distribution of the η_{sq} by

$$\eta_{s.} = A\omega_{s.} \ (each\ s) \tag{4}$$

where *A* is a lower-triangular $p \times p$ matrix. The elements of *A* are additional parameters in the model that can be included in a likelihood calculation.

Based on the structure described above, we will define a likelihood that incorporates all available monitoring data for each of the *p* pollutants, and we will estimate the parameters using maximum likelihood (see e.g. Szpiro et al.[2]). We will also explore Bayesian estimation methods to determine if these approaches offer an advantage in terms of computational burden or robustness of parameter estimates. Given a set of parameter estimates and the monitoring data, we will predict $\hat{X}_{s_i q}$ for each subject.

We can use the predicted multivariate exposure in separate disease models for each pollutant. We will conduct such analyses, but we are also interested in assessing multi-pollutant mixtures, and this requires constructing a disease model with *p* exposures and allowing for high-dimensional effect modification. Furthermore, since the *p* components of the predicted exposure are likely to be correlated with each other and subject to measurement error, there is a possibility of confounding between components even if they are all included in the same disease model.[13] Instead, we will perform principal component analysis (PCA) on $\hat{X}_{s_i q}$ and retain only the most important p' components as a way of reducing the dimension of the exposure. In addition to traditional PCA, we will consider defining the principal components based on eigenvalues of the coregionalization matrix *A* from our co-kriging model.[17] After projecting into the p'-dimensional principal component space, we will have a new predicted concentration field $\hat{X}_{s_i q'}$ for each subject. The derivation by PCA guarantees that the p' components of $\hat{X}_{s_i q'}$ will be nearly orthogonal, mitigating concerns about confounding related to measurement error. In addition, we will limit p' to a small number such as 2 or 3 so that we can feasibly propose a multivariate disease model that includes interaction terms for to account for effect modification. For a longitudinal outcome expressed as the difference between continuous outcome measurements obtained at two follow-up exams, with $p' = 2$ such a model will take the form

$$Y_i = \beta_0 + \hat{X}_{s_i 1}\beta_1 + \hat{X}_{s_i 2}\beta_2 + \hat{X}_{s_i 1}\hat{X}_{s_i 2}\beta_{12} + Z_i\beta_Z + \varepsilon_i \tag{5}$$

where Z_i represents additional subject-specific covariates. We can use an analogous Cox model for survival outcomes, and we can generalize the continuous outcome model for more general longitudinal data by incorporating random effects for intra-subject correlation.

In order to account for measurement error and ensure valid inference for the coefficients in equation (5), we will adapt the parametric bootstrap method recently developed for univariate exposures.[14] Since we do not need to be concerned about confounding induced by measurement error, it is straightforward to estimate the underlying data-generating mechanism

for $\hat{X}_{s_i q'}$ and Y_i based on the available data and then simulate multiple bootstrap samples in order to derive empirical estimates of bias and standard errors. For a Cox model, we will estimate a baseline hazard function to be incorporated in the bootstrap data-generating mechanism, and for longitudinal outcomes we will include estimated subject-specific random effects.

We cannot directly interpret the coefficients from (5), but we can use them to make inference about the relative risks of two arbitrary multi-pollutant mixtures, subject to approximation by projection into the principal component space. Suppose that we are interested in the relative risk of pollutant mixtures $c_1 = (c_{11}, \ldots, c_{1p})$ and $c_2 = (c_{21}, \ldots, c_{2p})$. Assuming $p' = 2$, we will project each of these into the principal component space to obtain $c_1' = (c_{11}', c_{12}')$ and $c_2' = (c_{21}', c_{22}')$ and then calculate the relative risk

$$RR_{12} = \hat{\beta}_1(c_{11}' - c_{21}') + \hat{\beta}_2(c_{12}' - c_{22}') + \hat{\beta}_{12}(c_{11}'c_{12}' - c_{21}'c_{22}'). \qquad (6)$$

We will obtain confidence intervals for the relative risk based on the parametric bootstrap standard errors for $\hat{\beta}_1$, $\hat{\beta}_2$, and $\hat{\beta}_{12}$. We will verify that this is an appropriate estimate of the relative risk for c_1 and c_2 by confirming that both pollutant mixtures are well approximated by their projections into the principal component space. This condition is expected to hold as long as c_1 and c_2 represent relevant pollutant mixtures in the sense of being similar to the combinations of pollutions observed in the atmosphere.

Objective 4: Identify additional statistical methodological research that will advance Center projects

The active participation of statisticians in the scientific research of the Center enables statistical methods projects to be identified. Ongoing activities in this Core will enable Center statistical investigators to identify innovative approaches to statistical analyses and statistical methods projects with the most pressing scientific need. Possible innovation will be two-way with non-statistician investigators providing motivating applications for statistical methods research and statistical researchers suggesting potential enhancements to Center-sponsored research. While additional methodological research (beyond the methods proposed in Objective 3) is not planned to be funded through the CCAR, project identification is the first step towards solving methodological problems. While we anticipate project identification will be followed by solicitation of resources for new statistical methods research, CCAR funds will not be used for this purpose.

Objective 5: Communicate and disseminate Center findings

(Bio)statistical expertise can contribute to dissemination of research findings in two important ways. First by aiding in the interpretation and communication of statistical analyses, research conducted by life science experts benefits from the most current understanding in the fields of statistics and biostatistics. Second, examples from CCAR research can be featured in statistical papers that a) provide new methodological development, or b) demonstrate methods implementation. Dissemination and communication will be supported through the following activities:

i. *Support manuscript review and preparation*, including interpretation of study results, manuscript writing, and response to reviewers;

ii. *Foster understanding of statistical methods*. This will be accomplished through ongoing interaction with Center investigators and preparation of papers discussing statistical methods implementation for life science journals.

iii. *Engage statisticians in air pollution research*. This will be accomplished by incorporating examples from CCAR research into (bio)statistical presentations and methodological papers.

3. ***Expected Results, Benefits, Outputs, Outcomes***

Provision for the Biostatistics Core demonstrates our belief that thorough and continuous involvement of statisticians from the design phase through the data analysis phase of studies is an essential component of the strength, quality, impact, and recognition of the Center for Clean Air Research. There are two primary outcomes provided by this Core: 1) to enhance the statistical rigor of all research conducted in all five projects of this proposal and thus improve their impact on scientific understanding and clean air policy, and 2) to advance understanding of methods for making inference about multi-pollutant mixtures in epidemiologic studies; this will result in reduced uncertainty in health risk assessments, and better, more targeted clean air policy. The outputs will be papers in the peer-reviewed literature, both in the content-specific literature (e.g. exposure, toxicology, epidemiology) and in the statistics literature, as well as new statistical methodology with accompanying computer code to implement this methodology, and predictions of pollution exposure for MESA Air subjects that can be incorporated into this and other epidemiologic cohort studies.

4. ***General Project Information***

The Biostatistics Core activities include statistical consultation, advice on database management and compilation, statistical analysis, statistical methods development, and dissemination. This Core will follow an active consultation model and it will take a proactive approach to statistical collaboration in Center research. It supports all Center projects to varying degrees depending upon anticipated need.

Role of the Biostatistics Core as a Resource: This Core will take a proactive approach to statistical guidance and collaboration by striving to support the statistical activities of all projects. This will occur through regular meetings, methodology development, and responses to requests. Our vision for Core support of projects, as summarized by percent total Core FTE effort over 5 years is presented in the following table.

Project Number	Project Director	Percentage over 5 Years (years 1-3; 4-5)
1	Yost	25%; 20%
2	McDonald	10%; 15%
3	Campen	10%; 15%
4	Kaufman	10%; 15%
5	Vedal	45%; 35%

All projects will be supported with respect to design, statistical analysis, advice on database management, identification of new statistical methods development, and dissemination objectives of this Core. Our budget includes specific plans for methods development in the first three years of funding; Projects 1 and 5 will be supported with this methodology development as is reflected in the greater percentage effort devoted by the Core to these projects in years 1-3.

Facility: The Core will be housed administratively in the Biostatistics Department with some staff located near many of the CCAR investigators. This co-location will facilitate close and ongoing interactions with all the Center projects and the Administrative Core. Facilities include computers for data storage and statistical analysis, as well as adequate office space to house this equipment and the Core staff.

Access: Access to the Biostatistics Core will be through numerous channels: a) Each project will have a designated staff liaison (Dr. Oron) who will be responsible for day-to-day activities. b) Each project will be invited to bring its statistical questions to one of the regular Core meetings. All faculty attend these meetings and a great deal of problem-solving occurs at that time. Projects that don't initiate contact on a semi-regular basis will be approached by the Core director to determine ways of establishing this contact. c) Each project will have a designated location on the server to store data and analysis files. Standard protocol will guide file documentation.

Structure: The Core will conduct its activities through staff effort devoted to individual projects and faculty effort devoted to staff oversight, methods development, and statistical consulting. The Biostatistics Core director, Dr. Sheppard, will oversee all the work of this Core. Methods development will be done in the first three years of funding by Dr. Szpiro and the postdoctoral fellow, with active collaboration by Drs. Sheppard and Sampson. The Biostatistics Core will hold regular meetings (typically weekly in conjunction with other related research) to discuss ongoing work and consult with projects. As needed, the Core director will initiate bi-yearly meetings to establish connections and support for individual projects. We expect a primary staff effort to concentrate on routine needs of the Center through its focus on data, data analysis, consulting, and interpretation of study results. Dr. Oron and the postdoctoral fellow (in years 4 and 5) will be assigned to individual projects to support their data and statistical needs; they will ensure there is sufficient communication between them that they can cover for each other. Project investigators have the discretion to conduct their own analyses (with advice as needed from this Core) or request Core staff to conduct the analyses for them. Biostatistics Core activities will be guided by clearly documented procedures regarding access to data and statistical assistance. All analyses performed by Core staff will follow a pre-specified analysis plan as documented in a SAP.

The primary faculty effort on this Core will be to develop methods to support projects 1 and 5, and to provide statistical expertise to ensure the integrity of all projects through collaboration and consultation. Multi-disciplinary collaboration will be coordinated during the regular Core meetings as well as during project-specific meetings.

Skills: The Core faculty have significant experience working in the air pollution field and with environmental data. Dr. Sheppard co-led the Biostatistics and Data Management Core for the EPA Northwest PM Center for five years. That activity resulted in direct contribution by one or more PM Center Core members to over 40 applied and methodological publications. Dr. Sampson has spent many years modeling air pollution fields and also leads the Statistical Consulting Program at the University of Washington Department of Statistics. Dr. Szpiro has focused for the last several years on implementing spatial and spatio-temporal models of air pollution concentrations and on developing novel statistical approaches to account for measurement error when incorporating predicted exposures in health effect analyses. Here are brief faculty bios:

Elizabeth A (Lianne) Sheppard is Professor in the Department of Biostatistics with a joint appointment in Environmental and Occupational Health Sciences. Her research interests focus on statistical methods for environmental and occupational epidemiology and include study design and estimation of environmental exposure effects. She actively collaborates with Department of Occupational and Environmental Health Sciences faculty, she is principal investigator on an EPA-funded cooperative agreement to incorporate exposure assessment into epidemiological study inference, and she is a co-investigator on several currently funded air pollution studies including the MESA Air Pollution Study, the NIEHS-funded DISCOVER Center to study the effects of traffic-related pollution on cardiovascular disease, and the HEI-funded NPACT study of air pollution components and health. By leveraging this and other research funding, she has mentored 12 biostatistics students as they became engaged in environmental health applications. She has served as advisor or committee member for 11 PhD students in six departments, mentored 2 post docs, and advised many additional Masters students. She is Principal Investigator of a recently funded NIEHS training grant to integrate quantitative with environmental health sciences. Dr. Sheppard was elected Fellow of the American Statistical Association in 2006 and she is or has been a member of two Clean Air Scientific Advisory Committee Special Review Panels, the HEI Review Committee, and the Advisory Committee for the USC Children's Study.

Paul D. Sampson, PhD, is Research Professor of Statistics and Director of the Statistical Consulting Program in the Department of Statistics. His primary research is on spatio-temporal and multivariate modeling for environmental processes. He has also developed and applied morphometric and other multivariate methods in the context of studies of Fetal Alcohol Syndrome at the Fetal Alcohol and Drug Unit. As a co-investigator on MESA Air study, he is developing a spatio-temporal model for air pollution concentration based on a combination of sparse spatial dataset with rich temporal sampling and a relatively rich sampling of spatial location with few time points. This model is also the basis of the assessment of the design of the MESA Air monitoring networks.

Adam Szpiro, PhD, is Assistant Professor in the Department of Biostatistics. He is currently working on statistical methodology for air pollution research as part of MESA Air as well as several other studies funded by NIEHS, EPA, and the Health Effects Institute. A major focus of Dr. Szpiro's research is developing spatio-temporal models to predict ambient air pollution concentration fields that exploit sparse data and account for complex spatio-temporal dependencies in a computationally efficient framework. He is also developing novel statistical

approaches to account for measurement error that results from using predicted air pollution values as the exposures in health effect analyses. In addition to his work in environmental epidemiology, Dr. Szpiro is working on extending the theory of model-robust inference in a way that bridges the conceptual gap between Bayesian and frequentist reasoning.

Figure: Statistical Analysis Plan Template

Working Title:

Overview/Purpose:

General Scientific Question(s):

Specific Scientific Question(s) (e.g. hypotheses):

Outcomes of Interest:

Predictors of Interest:

Potential Confounders or Adjustment Variables:

Other Data Specifics (e.g. time period, subgroup):

Data request (date, number):

Type of Analysis:

Hypothesis testing		Estimation
Hypothesis screening		Modeling
Hypothesis generating/exploratory		Method evaluation
Descriptive		

Analysis Approach and Special Issues:

List of Tables: (or note location of draft tables)

Plan of Action:

Responsibilities and deadlines:

- Paper outline
- Initial analyses
- Introduction
- Methods
- Results
- Discussion
- Tables and Figures
- Follow up analyses
- Final Draft

Names and roles (authors, co-authors, Data Core staff):

Revision History: