# Central Nervous System Effects of Ambient Particulate Matter: The role of Oxidative Stress and Inflammation

# **Final Report**

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Agency

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# List of Inventions

None

# List of Copyrighted Materials

None

# List of Terms, Abbreviations and Symbols

Symbol or Abbreviation	Explanation					
AAALAC	Association for the Assessment and Accreditation of Laboratory Animal Care					
APHEL	Air Pollution Health Effects Laboratory, University of California, Irvine					
apoE-/-	Mice in which the gene regulating apoE is deleted, or "knocked out"					
AQMD	Southern California Air Quality Management District					
Arteriosclerosis	A chronic disease in which deposits of cholesterol and/or calcium cause abnormal thickening and hardening of the arterial walls with resulting loss of elasticity					
CAPs	Concentrated Ambient Particles					
EC	Elemental Carbon					
ECG	Electrocardiogram					
FA	Filtered Air (Air filtered through a High Efficiency Air Filter; HEPA)					
GSH	Reduced Glutathione					
GSSG	Oxidized Glutathione					
HNE	Hydroxynonenal (Marker of Lipid Peroxidation)					
IL-6	Interleukin-6 (Inflammatory Cytokine)					
IL-10	Interleukin-10 (Inflammatory Cytokine)					
MDA	Malondialdehyde (Marker of Lipid Peroxidation)					
MSU	Michigan State University					
NF-ĸB	Nuclear Factor-KB (Inflammatory Signaling Agent)					
NYC	New York City, Mount Sinai Hospital Exposure Site					
NYU	New York University					

OC	Organic Carbon
PM	Particulate matter
PM <sub>2.5</sub>	Particulate matter less than 2.5 µm in aerodynamic diameter
SEA	Seattle, University of Washington, Exposure Site
SF	Sterling Forest, New York, Exposure Site
ΤΝFα	Tumor Necrosis Factor-a (Inflammatory Cytokine)
VACES	Versatile Aerosol Concentration Enrichment System
UCI	University of California, Irvine, Exposure Site

# Abstract

The objective of this project was to determine how the biological responses in the brains of apoE-/- mice exposed to fine PM (PM that is 2.5 microns or less in diameter, PM2.5) might depend on the composition of ambient PM emitted from vehicles, power generation, industrial processes and other sources. Brain tissue from mice exposed to concentrated ambient fine particles (CAPs) was obtained by collaborative agreements from a multicity study that was part of an ongoing program sponsored by the Health Effects Institute and conducted by Dr. Morton Lippmann and his colleagues at New York University (NYU). Mice were exposed in New York City (NYC), Sterling Forest, NY (SF), Seattle, WA (SEA), East Lansing, MI (MSU) and Irvine, CA (UCI). Exposures were 6 hours per day, 4 days per week for 26 weeks (6 months). Brains were harvested and analyzed for inflammatory cytokines (Interleukin-6, Interleukin-10 and Tumor Necrosis Factor- $\alpha$ ), reduced and oxidized glutathione, as markers for anti-oxidant defenses, and biomarkers of oxidative stress (protein carbonyls, hydroxynonenal and malondialdehyde). Particulate samples from the exposure atmosphere were collected and analyzed for mass concentration, elemental and organic carbon contents and trace metal composition. The component data were used in a source apportionment analysis to identify the key sources that contributed to the PM2.5 at each of the five locations. While the analysis of the trace component composition and the source apportionment determinations provided useful confirmation of the *a priori* criteria used for selecting the exposure locations used in this study, differences in exposure concentrations, times of study and differences in the efficiency of operation of the VACEs between the five locations made it difficult to draw strong conclusions about source contribution influences on brain outcomes. This study demonstrated that exposure of apoE-/- mice to CAPs was associated with inflammatory changes in the brain, and that on a regional basis, the sections of the brain that were lower in the signal transducer nuclear factor-KB  $(NF-\kappa B)$  tended to be more susceptible to inflammatory changes. In addition the levels of NFκB decreased as the animals aged during the 6 month study in both Filtered Air (FA) and CAPsexposed mice. Sections of the brain with lower NF-KB levels also tended to exhibit exposurerelated increases in concentrations of biomarkers of oxidative stress, consistent with a correlation between inflammation and oxidative changes in the brain. Oxidative changes in the brain were

consistently observed in mice exposed at SEA but not in mice exposed at MSU. Mice exposed at UCI showed a pattern of changes that was the same as that seen in SEA mice but because the UCI brains had been sectioned for brain regional analysis, the variances were larger in the UCI group than in the SEA group and the average effect differences compared to FA-exposed brains did not achieve statistical significance. The sources of PM at the UCI and SEA sites were more influenced by emissions related to oil combustion (such as motor vehicles, power generation, space heating) than was the PM at the MSU site, as evidenced by a high correlation of PM concentrations with the concentrations of Ni and V in the particles at UCI and SEA but not at MSU. The concentrations of Ni and V in the exposure atmosphere were probably too low to be directly toxic and it is likely that these elements are tracers or surrogates for oil combustion aerosols which would include EC, OC and BC. The pattern of more exposure-related increases in oxidative stress markers at SEA and UCI relative to MSU could indicate that the products of oil combustion from mobile, power generation and space heating sources may be important factors in the inflammatory and oxidative changes noted in the brains of apoE-/- mice exposed to CAPs.

## **Executive Summary**

## Background

We and others have shown that the concentrations of the activated forms of signal transduction proteins, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NfkB) and mitogen activated protein kinases (MAPK's), are elevated in the brains of mice exposed to concentrated fine (PM2.5, particles smaller than 2.5 µm in aerodynamic diameter) and 'ultrafine' (particles smaller than 0.2 µm in aerodynamic diameter) ambient particles (CAPs) in areas near primary emission sources. In addition, biomarkers of oxidative stress and tissue injury are observed at higher concentrations in the brains of mice after exposure to fine and 'ultrafine' CAPs. The objective of this project was to determine how the biological responses related to inflammation and oxidative stress in the brains of genetically modified mice (apoE-/-mice in which the apoE gene is deleted thus resulting in impaired metabolism of lipids and cholesterol in these mice) exposed to fine PM might depend on the composition of ambient PM emitted from vehicles, power generation, industrial processes and other sources.

#### Specific Aims

The aim of this study was to determine whether PM-induced biochemical changes in the central nervous system (CNS) are influenced by PM concentration, source or compositional differences. *In vivo* biological responses, which have been identified as biomarkers of CNS exposure or injury, were examined with respect to the physical and chemical composition differences between the PM from five sites with distinctly different ambient PM sources. This study provided a unique opportunity for resource leveraging because the very costly and involved exposure and atmosphere characterization phases were funded by a multimillion dollar HEI grant.

#### Methods

The study was conducted in five locations with different PM2.5 source profiles. Mice were exposed in New York City (NYC), Sterling Forest, NY (SF), Seattle, WA (SEA), East Lansing,

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MI (MSU) and Irvine, CA (UCI). NYC is a densely populated urban environment with major impacts from traffic and oil-fired power generation. SF is a rural location with regional source contributions. The SEA location is on a university campus with moderate levels of traffic but there is a strong regional influence of wood burning for space heating during the winter months. MSU is an urban area which is influenced by coal burning sources and is more industrial than the other locations. UCI is a suburban area with major impacts from nearby heavily trafficked roads and regional influence of diesel emissions associated with operations at the ports of Long Beach and Los Angeles. Genetically modified male 6 week old mice (apoE-/-) were exposed to filtered air (FA) or concentrated ambient PM<sub>2.5</sub> particles (CAPs) for 6 hr per day, 4 days per week for 26 weeks at each of the five locations. ApoE-/- mice have reduced ability to metabolize lipids and therefore have very high levels of circulating lipids and cholesterol. Some of the mice were implanted with telemetry devices so that ECG parameters could be monitored while the mice were exposed. At the end of the 26 week exposure brains were harvested and analyzed for biomarkers of inflammation and oxidative stress. Air samples collected during the exposures were analyzed to provide elemental composition data which were used to apportion the sources of PM2.5 in the five locations.

#### Results

The source apportionment analyses agreed with the *a priori* assessments of the key sources that contributed to the PM2.5 at each of the five locations. Exposure of apoE-/- mice to CAPs was associated with inflammatory changes in the brain and that on a regional basis, the sections of the brain that were lower in the signal transducer NF- $\kappa$ B tended to be more susceptible to inflammatory changes. In addition, the levels of NF- $\kappa$ B decreased as the animals aged during the 6 month study. There also appeared to be an exposure-related increase in concentrations of biomarkers of oxidative stress. These increases in exposure-related oxidative stress were observed primarily in the sections of the brain with lower NF- $\kappa$ B levels, consistent with a correlation between inflammation and oxidative changes in the brain.

## **Conclusions**

This study demonstrated that exposure to CAPs from some locations can induce inflammatory changes and oxidative stress in the brains of apoE-/- mice. Significant effects were seen in the mice exposed at SEA and UCI but not in mice exposed at MSU. A different suite of assays had been applied to the mice exposed at NYC and SF. Those exposures had been performed several years prior to the inception of this ARB-funded study and the results were published separately. Notable findings were that there were exposure-related increases in brain inflammation, evidenced by increased levels of glial-fibrillary acidic protein (GFAP, a protein released by innate immune system brain cells that are associated with brain inflammatory processes) and by the decrease in the numbers of brain cells that stain positive for the enzyme tyrosine hydroxylase, which are the dopamine-producing cells in the part of the brain called the substantia nigra. Loss of dopamine-producing cells is a hallmark of Parkinson's disease [1, 2].

The failure to see significant changes in the MSU mice may have been due to a high level of background inflammation and oxidative stress in the mice used at that site since the concentrations of relevant biomarkers were higher for the FA-exposed mice at that location than for FA-exposed mice at UCI or SEA. The baseline levels of NF-KB increased in the brains of FA and CAPs-exposed mice as one progressed from the rostral to the mid-brain to the caudal regions, and NF-KB levels decreased as the mice aged from 2 to 4 to 6 months from the start of the exposure study. Protein carbonyl concentrations in the cytoplasmic fractions of rostral, midbrain and caudal sections of air-exposed and CAPs-exposed mice were significantly higher in the rostral sections in both air- and CAPs-exposed mice, however at 2 months of exposure there was a significant increase in rostral sections from CAPs-exposed mice compared with levels in the air-exposed group. After 6 months of exposure, however, the protein carbonyl concentrations in the rostral sections were not significantly different between air-exposed and CAPs-exposed mice but there was a significant (albeit small) increase in the mid-brain sections. The rostral section has lower NFKB activation levels than either the midbrain or caudal sections, so the finding of significant protein carbonyl increases in regions with lower NF-KB levels is consistent with a role for NFkB in antioxidant defenses of the brain.

Oxidative changes in the brain were consistently observed in mice exposed at SEA but not in mice exposed at MSU. Mice exposed at UCI showed a pattern of changes that was the same as that seen in SEA mice but because the UCI brains had been sectioned for brain regional analysis,

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the variances were larger in the UCI group than in the SEA group and the average effect differences compared to FA-exposed brains did not achieve statistical significance. While the analysis of the trace component composition and the source apportionment determinations provided useful confirmation of the *a priori* criteria used for selecting the exposure locations used in this study, differences in exposure concentrations, times of study and differences in the efficiency of operation of the VACEs between the five locations made it difficult to draw strong conclusions about source contribution influences on brain outcomes. The sources of PM at the UCI and SEA sites were more influenced by emissions related to oil combustion (such as motor vehicles, power generation, space heating) than was the PM at the MSU site, as evidenced by a high correlation of PM concentrations with the concentrations of Ni and V in the particles at UCI and SEA but not at MSU. The concentrations of Ni and V in the exposure atmosphere were probably too low to be directly toxic and it is likely that these elements are tracers or surrogates for oil combustion aerosols which would include EC, OC and BC. The pattern of more exposure-related increases in oxidative stress markers at SEA and UCI relative to MSU could indicate that the products of oil combustion from mobile, power generation and space heating sources may be important factors in the inflammatory and oxidative changes noted in the brains of apoE-/- mice exposed to CAPs.

# Central Nervous System Effects of Ambient Particulate Matter: The role of Oxidative Stress and Inflammation

# Background

The brain is a potential target for adverse effects after inhalation exposure to particulate matter (PM). The U.S. Environmental Protection Agency has established a national ambient air quality standard for PM2.5, defined as particles with an aerodynamic diameter  $\leq 2.5 \,\mu$ m. PM2.5 actually contains a mixture of different sized particles including particles in the ultrafine ( $\leq 0.2$  $\mu$ m aerodynamic diameter) and nanoparticle ( $\leq 0.1 \mu$ m aerodynamic diameter). Kreyling and others [3-5] demonstrated that inhaled, nanosize particles could leave the lungs and be deposited in extra-pulmonary tissues, such as the liver and the brain. It is also possible that dissolved chemical from inhaled particles deposited in the lung or mediators released in the lung by inflammatory or oxidative processes could be transported to the bloodstream and affect distal organs including the liver, the heart and the brain. We and others have shown that the concentrations of the activated forms of signal transduction proteins, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NfkB) and mitogen activated protein kinases (MAPK's), are elevated in the brains of mice exposed to concentrated fine and ultrafine ambient particles (CAPs) in areas near primary emission sources [6, 7]. NfkB is a protein complex that when activated migrates into a cell nucleus and controls transcription of genes that mediate inflammatory responses. In addition, biomarkers of oxidative stress and tissue injury are observed at higher concentrations in the brains of mice after exposure to fine and ultrafine CAPs for as long as 2-weeks post-exposure [6]. A major gap in the existing knowledge base is the degree to which neurotoxicity in the central nervous system (CNS) is dependent on particle dose and particle composition. This project took advantage of an opportunity to leverage an ongoing multi-city HEI-funded study to address this data gap.

Our previous studies of mice [8] and rats [9, 10] exposed to fine and ultrafine PM in southern CA cities showed that these exposures had significant adverse cardiovascular effects resulting in increased rates of formation of atherosclerotic-like plaques and changes in cardiac function. It should be noted that these studies were performed using apoE-/- mice which were genetically susceptible to developing atherosclerosis. Normal, or wild-type, mice would be unlikely to exhibit the same effects from the 8-week exposure. Our *in vivo* inhalation studies of airway allergies near freeways suggested that the greatest biological activity was associated with the fraction of PM that contained the greatest amounts of elemental carbon (EC) and organic carbon (OC) compounds [11, 12], suggesting that PM-induced toxicity was a function of particle size distribution and composition.

ApoE is a glycoprotein that is synthesized mostly in the liver and brain but is also produced by macrophages and monocytes [13]. It acts as a ligand for receptors that clear chylomicrons and very low density lipoprotein fragments and is thought to mediate cholesterol homeostasis and function in absorption and biliary excretion of dietary cholesterol. ApoE-/- mice are widely used in studies of atherosclerosis [13]. The genetically modified mice (apoE-/-) used for our cardiovascular disease studies are also a well-recognized model for CNS neurotoxicity. ApoE is a protein that can decrease microglial activity and TNF- $\alpha$  secretion, thus attenuating inflammatory responses in the brain [14]. ApoE is the major cholesterol transporter in the brain, and human carriers of the e4 allele of apoE are at a higher risk of developing Alzheimer's disease[15]. It is also known that the brain of the apoE-/-mouse is more susceptible to oxidative stress than is the wild type background strain mouse. In the hippocampus, the absence of ApoE altered oxidant/antioxidant status and the endogenous level of thiobarbituric acid-reactive substances (TBARS) was markedly elevated whereas the level of alpha-tocopherol was decreased in ApoE-/- mice compared to wild-type mice [16-18], consistent with a pro-oxidant state. This might have been mediated via activation of glial cells. Compared to wild type mice, glial cells cultured from ApoE-/- mice exhibit an enhanced production of several proinflammatory markers in response to treatment with amyloid- beta and other activating stimuli [19]. ApoE deficiency also accelerates the age-related decline in efficacy of the blood brain barrier [20, 21]. Thus, the apoE deficiency induced in the apoE-/- mutant may result in increased susceptibility to cerebral inflammation, especially since this mutant exhibits chronic systemic inflammation [22]. These characteristics of the apoE-/- mice suggested that these mice would potentially be susceptible to the effects of CAPs exposure, and could, in addition to having increased oxidative stress and cardiovascular effects, also exhibit heightened levels of oxidative stress and errors in modulating oxidative stress in the brain.

In our earlier studies, we found that in addition to cardiopulmonary effects, there was significant up-regulation of the immune-related transcription factors NF- $\kappa$ B and AP-1, as well as increased concentrations of two principal pro-inflammatory cytokines, IL-1 $\alpha$  and TNF- $\alpha$ , in the brain after exposure to CAPs present in air pollution [23, 24]. NF-KB promotes the expression of genes involved in inflammation, such as proinflammatory cytokines and inducible nitric oxide synthase (iNOS). In addition our studies suggest that these effects may be mediated by mitogen activated protein kinase (MAPK) pathways [24]. In mice that were similarly exposed to fine CAPs in NY, glial fibrillary acidic protein (GFAP) staining, which is highly expressed in the macrophage-like astroglial cells of the brain, was increased significantly, and there was evidence of degeneration of dopaminergic neurons leading Peters and colleagues to draw the following conclusion. "Morphometric analysis of the CNS indicated unequivocally that the brain is a critical target for PM exposure and implicated oxidative stress as a predisposing factor that links PM exposure and susceptibility to neurodegeneration. Together, these data present evidence for potential translocation of ambient particles on organs distant from the lung and the neurodegenerative consequences of exposure to air pollutants." [25]. Brain tissue from mice exposed to concentrated ambient fine particles (CAPs) were obtained by collaborative agreements from a multicity study that was part of an ongoing program sponsored by the Health Effects Institute and conducted by Dr. Morton Lippmann and his colleagues at New York University (NYU) to investigate the role of PM composition and concentration on development and exacerbation of cardiovascular disease. Exposures had already been conducted in urban New York City (NY) and rural Sterling Forest which is in Tuxedo, NY (SF) and brain samples from those two locations had been previously assayed [2, 25]. This present ARB study provided a unique opportunity to examine the role of fine PM on the brain at three additional exposure sites, and also to examine whether local differences in PM sources or chemistry were important modifiers of biochemical changes in the CNS.

## **Overview of New York and Sterling Forest Studies**

Samples of brains of CAPs and FA exposed mice at NY and SF sites (6–9/treatment) were embedded and serially sectioned in the coronal plane. Slides were immunohistologically stained for markers of oxidative stress (iNOS, heme oxygenase-1) and markers of neurotoxicity (tyrosine hydroxylase (TH), a marker for dopamine containing neurons and glial fibrillary acidic protein (GFAP), a marker of astrocytic proliferation) and 8-hydroxyguanosine, an oxidized nucleic acid base that is a marker for oxidative stress. These data were published by NYU and a summary of the results is presented in our results section. Frozen brain tissue, 6-9 samples from each exposure atmosphere, were processed and Western blots were run and analyzed for the transcription factor NF- $\kappa$ B. Extracted tissue was analyzed for inflammatory cytokines (IL-1, IL-6, TNF $\alpha$ ), biomarkers of antioxidant defenses (reduced and oxidized glutathione, protein carbonyls), and two biomarkers of lipid peroxidation (malondialdehyde and hydroxynonenal).

The objective of this project was to determine how the biological responses in the brains of apoE-/- mice exposed to fine PM might depend on the composition of ambient PM emitted from vehicles, power generation, industrial processes and other sources. This study addressed the question of whether PM-induced biochemical changes in the CNS are influenced by PM concentration, source or compositional differences. *In vivo* biological responses, which have been identified as biomarkers of CNS exposure or injury, were examined with respect to the physical and chemical composition differences between the PM from five sites with distinctly different ambient PM sources. This study provided a unique opportunity for resource leveraging because the very costly and involved exposure and atmosphere characterization phases are funded by a multimillion dollar HEI grant.

# Methods and Materials

## **ARB-funded Study Sites**

Brain tissue was obtained and analyzed with ARB support after HEI-funded exposures that were conducted on the campuses of Michigan State University in East Lansing, MI (air pollution influenced by emissions from industrial sources as well as oil and coal burning power generation), the University of Washington Seattle, about 8 miles north of downtown Seattle (a site with moderate traffic and a major influence from wood smoke emissions from space heating during the winter), and the University of California, Irvine (primary contributions from regional and traffic-related sources). The Michigan site was near Michigan State University in East Lansing, MI (pop 46,420) which is part of the Lansing Metropolitan Area (pop 453,603). A 61 megawatt (MW) and a 351MW coal burning power plant are major emission sources that could impact the exposure site. In addition there are some medium to light industries, including automotive assembly plants (General Motors), steel (welding and fabricating), and metal processing facilities. The site could also be affected by regional emission sources in the Midwest which could include transported particles emitted by coal-fired power plants, industrial activities and agricultural enterprises. The Irvine site, which is within the Los Angeles basin (LAB) is proximate to heavily trafficked roadways with some influence from regional coastal sources that could include emissions from oil refineries and operations at the Ports of Long Beach and Los Angeles when winds are from the north and west.

The basic methods for exposure and brain processing were the same at all five sites, and are described below. Briefly, brains from mice exposed to fine CAPs for 6 months at each study site were examined using biochemical, immunohistochemical and molecular biological methods. In addition, shorter term exposures (2 months and 4 months in Irvine, Ann Arbor and Seattle; 3 months and 6 months in NYC and Tuxedo) provided some data relevant to development and progression of cardiovascular and neurotoxic effects. All of the exposures used apoE-/- genetically modified mice that were exposed to CAPs at each site using a pre-agreed protocol and identical exposure systems. For all of the sites in this study, an *in-vivo* rodent exposure system, based on the system designed at UC Irvine [26] and the VACES particle concentrator that was designed by Sioutas and colleagues [27] were used. The VACES was used to increase the concentration of ambient aerosol to approximately 10 times ambient at each of the study sites, but there were differences in ambient concentrations that made it difficult to match particle mass and/or number concentrations among sites. The exposure systems were provided by NYU for each site and exposure methods and protocols were the same, to the extent possible, at each site.

Atmospheric PM2.5 samples were monitored during exposures at each site and were analyzed for particle mass concentration and for elemental carbon (EC) and organic carbon (OC). In addition sample from the VACES were analyzed for EC, OC and inorganic constituents by Dr. Lung Chi Chen and colleagues at NYU. Samples of brains (n=4-12 from FA

and CAPs-exposedmice at each location were prepared and evaluated as described below. CNS outcomes were examined in the mice using tissue collected 1 day after the last exposure to CAPs or filtered air (FA).

#### **Experimental Techniques**

#### Selection and Characteristics of the Animal Model

The transgenic mouse model of cardiovascular disease that we used in this study was developed from the C57BL/6 mouse. The C57BL/6 strain, which lacks the apolipoprotein E receptor (apoE-/-), has been shown to be particularly susceptible to cardiovascular effects but is also subject to adverse pulmonary effects from a variety of inhaled substances including  $O_3$  [28], acid-coated carbon particles [29], ovalbumin [30] and concentrated pseudo-ultrafine particulate matter (PM<sub>0.18</sub>) [8]. As mentioned earlier this strain is also prone to developing inflammatory changes in the brain since apoE has anti-inflammatory properties and the lack of this protein could be proinflammatory [31].

#### Animal Husbandry

Male apoE-/- 6 week old mice were purchased from a commercial supplier and housed two to a cage in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited animal housing facility at the Air Pollution Health Effects Laboratory (APHEL). All animals used in this study were apoE-/- mice and each exposure group consisted of 16 mice. Mice were exposed to FA or CAPs for 2 months, 4 months and 6 months. Six FA and six Caps mice were implanted with telemetry devices were exposed for 6 months. The implanted mice were housed singly so that ECG parameters could be monitored while the mice were in the vivarium. While in housing the animals breathed filtered, purified air and were provided with food and water *ad libitum*. Telemeter-equipped mice were monitored during exposures and while they were in the vivarium, but were not monitored during loading and unloading to the exposure chambers. On the average, mice were monitored about 22 hr per day. ECG data from the same time periods were evaluated on exposure and non-exposure days so the unmonitored transport time did not impact our analyses.

#### **Ambient Particle Concentrator**

Ambient particles with particle diameters smaller than 2.5  $\mu$ m (PM2.5), including the ultrafine fraction of ambient PM, were concentrated using the Versatile Aerosol Concentration Enrichment System (VACES) which has been described in detail by Kim et al. [32, 33]. VACES consists of a PM2.5 size selective inlet, a saturator/chiller module that supersaturates the aerosol with water vapor causing fine and ultrafine particles to grow to a size that can be inertially separated using a virtual impactor, and a diffusion drier module that removes the excess water vapor and returns the aerosol to a size distribution that is very close to that in the unconcentrated ambient air (Figure 1). The system is mobile and capable of enriching the concentration of particles in the range of 0.03- 2.0  $\mu$ m by up to a factor of 30 x ambient, depending on the output flow rate [34]. The concentration efficiency falls off above 2.0  $\mu$ m or below 0.03  $\mu$ m.

#### **Exposure Chambers**

A whole-body exposure mouse chamber (Figure 2) was designed specifically for use with the VACES. Each stainless steel (SS) chamber (50 cm length × 27 cm width × 15 cm height) was segmented into 18 cubicles (1 mouse per cubicle) separated by perforated SS sheets (0.078" hole diameter, 36% open and staggered; McMaster-Carr, New Brunswick, NJ). Concentrated ambient particles (CAPs) were delivered through SS particle delivery tubes that distributed CAPs uniformly throughout the exposure chamber [35]. A raised sub-floor constructed from perforated SS sheet (0.25" hole diameter, 50% open, staggered) was used, which permitted urine and feces to fall to the bottom of the vat and kept the mice relatively clean. The exposure atmosphere was exhausted from below the sub-floor through 2 SS tubes, each 40 cm in length with 28 0.5 mm downward-facing holes. Absorbent sheets impregnated with an antibiotic to prevent fecal bacteria from generating ammonia from urine were placed under the exhaust lines to absorb urine and to collect feces. Figure 2 shows the rectangular stainless-steel pan, perforated stainless-steel floor, partitions, copper aerosol inlet and distribution pipe, and copper aerosol outlet. The aerosol return line below the floor is not shown. The aerosol inlet is designed to connect to the outlet of the VACES aerosol concentrator.



Figure 1. System for Exposing Mice to Concentrated Ambient Particles (CAPs).



Figure 2. Schematic diagram of the mobile exposure cage.

#### **Exposures**

During exposures, concentrations of ambient and CAPs particles were monitored. Samples were also collected on quartz filters that were pre-treated at 400°C to remove adsorbed organic compounds. These filters were composited on a weekly basis and analyzed for elemental carbon (EC) and organic carbon concentrations (OC). EC is a reasonable tracer for particles originating from diesels [36] and represents between 5 - 20% of the UFP in ambient samples. EC and OC were measured by a thermal photometric method on a fraction of the filter; the remaining fractions were stored (-80° C) and subsequently analyzed for trace constituent concentrations. Particles were also collected for mass concentration and chemical analyses on pre-weighed fluorocarbon filters. Following collection, the filters were equilibrated overnight at constant humidity and weighed. These filters were submitted for X-ray Fluorescence analysis of elemental constituents, among which were Fe, V, Zn, Cr, Ni, Cu, Pb and Mn. These specific metals are identified because previous inhalation or *in vitro* studies have shown them to be potentially toxic [37-43] and some were also useful tracers for emissions from specific sources. The full list of constituents measured is shown in Table 1.

#### **Exposure** Procedure

Animal exposure protocols were pre-arranged with the project organizer (NYU) and were virtually identical at each of the five sites. An exposure system was shipped by NYU to each exposure location and mice were obtained from the same supplier to minimize experimental variation between locations. The protocol used at UCI was representative of that used at each of the other sites, and will be described in this section.

The mice were housed in ventilated caging attached to an air purification system. The air purifier delivered filtered air at flows adequate to provide 15-20 air exchanges per hour in each ventilated cage unit. The vivarium was supplied with Class 100 filtered air using a laminar flow air purifier that consisted of a 1000 CFM blower, an oxidizing adsorbent canister containing permanganate-impregnated alumina spheres, and a high efficiency particle air (HEPA) filter system. Between exposures the animals were supplied with the described purified air, clean water and food, *ad lib*. Groups of 16 animals were placed into sealed, compartmentalized

exposure chambers and were exposed to concentrated fine PM2.5 particles (CAPs) for 6 hours per day, 4 days per week for 26 weeks. Control animals received purified air under conditions identical to those of the animals exposed to CAPs. Temperature was monitored continuously during the exposures and held to  $75 \pm 5$  °F. Animals were observed throughout the exposures for signs of distress (*e.g.* changes in grooming, food and water uptake, shaking). The Versatile Aerosol Concentrator for Exposure Studies (VACES) designed by Sioutas and colleagues at USC [32-34, 44, 45] was used for the PM exposures. This device has been used by UCI for exposures of mice near freeways at other locations in Los Angeles [46]. At approximately 8 weeks, 16 weeks and 26 weeks the mice were examined using an ultrasonic microscopy method, which will be described later in this section, to document the development of atherosclerotic



plaque in coronary arteries. The animals were euthanized 24 hr after the last ultrasonic measurements were made after the 26-week exposure period.

Figure 3.<sup>1</sup> The ultrasonic probe was used to measure the brachiocephalic (a1), left common carotid artery (a2), the left subclavian artery (a3) and the aortic arch (AA) for plaque.

<sup>&</sup>lt;sup>1</sup> Figure adapted from:

https://www.google.com/imgres?imgurl=http%3A%2F%2Fing.tfd.com%2Fdorland%2Fthumbs%2Farcus\_aortae.jp

#### Ultrasonic Microscopy

Ultrasonic microscopy was performed using a VEVO 660 UBM rolling cart developed by VisualSonics, Inc., which was provided and operated for this study by Dr. Chen and his colleagues from NYU at all five study locations. The unit provided a two-dimensional image with a spatial resolution of ~50  $\mu$ m, with penetration depth of ~20 mm. The NYU investigators used this device to measure arterial cross-sections prior to deploying the animals in the field and at 2 month intervals during exposures. This non-invasive method allowed us to monitor the development of atherosclerotic plaques in the vasculature. In addition we were also able to use Doppler echocardiography to measure hemodynamic parameters such as blood flow and cardiac ejection fraction. These measurements were repeated at about 8-10 week intervals during the exposures. The locations and identifications of the arteries measured are shown above (Figure 3).

#### **Bioassay and Data Analysis Methods**

#### **Brain processing**

For the CA exposures, whole brains were sectioned into *Rostral, Mid*, and *Caudal* regions (Figure 4) using a mouse brain matrix and sections were weighed prior to homogenization as per Bolon et al. [47]. For exposures in WA and MI, the right brain hemisphere was collected for each mouse and weighed prior to homogenization. Brain samples were homogenized and centrifuged, following the method of Lahiri et al [48], to obtain cytoplasmic, nuclear, and membrane subcellular fractions. Cytoplasmic fractions were used for cytokine, protein carbonyl and glutathione analyses. Nuclear fractions were used for NF- $\kappa$ B levels determination. Membrane fractions were used for the lipid peroxidation assay.

g&imgrefurl=http%3A%2F%2Fmedical-

dictionary.thefreedictionary.com%2Fdental%2Barch&docid=geC4EJbEgygtHM&tbnid=7VS3JmBq4XnaYM&w=2 50&h=195&ei=oCfLUu7PCYHyoASnloLQBA&ved=0CAMQxiAwAQ&iact=c.

# Anterior Posterior

Figure 4. Horizontal section through a mouse brain (adapted from Mouse Brain Atlas - http://www.mbl.org/atlas005/atlas005\_frame.html).

#### **Transcription factors**

NF- $\kappa$ B levels in the nuclear fractions of brain homogenates were determined using a gel shift mobility assay which makes it possible to differentiate activated NF $\kappa$ B from non-specific electrophoretic bands that might otherwise obscure the actual concentrations (Figure 5). The assay system used was developed by Promega (Madison, WI) as described by Campbell et al. [49].

#### Air CAPs Air CAPs Air CAPs

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Figure 5. Gel shift mobility assay showing the activation of NF-κB in tissue homogenates from the brain rostral region after 6 months of exposure.

#### Cytokines

The levels of IL-6, IL-10, MCP-1 and TNFα were measured in the cytoplasmic fraction of brain homogenates using a multiplex immunoassay (Millipore, Billerica, MA).

#### **Protein carbonyl assay**

NF-KB band

Protein carbonyl content in the cytoplasmic fraction of brain homogenates was determined using a fluorometric assay as described by Mohanty et al [50], in which fluorescein thiosemicarbazide (FTC) binds to protein carbonyl groups in a 1:1 ratio. Briefly, nucleic acids present in the cytoplasmic fraction were precipitated and removed prior to incubating the samples overnight with 200  $\mu$ M FTC. The proteins were then precipitated with 20% trichloroacetic acid (TCA) and the unbound FTC was removed by washing the protein pellet with acetone. After solubilizing the protein pellet with 6M guanidine hydrochloride, the fluorescence of the protein carbonyl-FTC product was measured with a 485<sub>em</sub>/530<sub>ex</sub> filter set.

#### Lipid peroxidation assay

The levels of malondialdehyde (MDA) and hydroxynonenal (HNE) were determined spectrophotometrically following a modified protocol developed by Gerard-Monnier et al. [51]. Briefly, 140  $\mu$ L of membrane fractions from brain homogenates were mixed with 455  $\mu$ L of

13mM of N-methyl-2-phenylindole followed by the addition of 105  $\mu$ L of concentrated HCl. The reaction was incubated in a water bath for 1 hour at 45°C. Each sample was centrifuged for 10 min at 10,000 g and the supernatant absorbance was measured at 586 nm.

#### **Reduced/oxidized glutathione**

Quantification of total (tGSH), reduced (rGSH) and oxidized (GSSG) glutathione levels were measured using the enzymatic recycling method [52]. Briefly, 5% sulfosalicylic acid was added to cytoplasmic fractions of brain homogenates to precipitate protein prior to glutathione determination. After centrifugation, supernatants were incubated for 5 min with a reaction mix containing 0.26mM NADPH, 0.74mM DTNB and 0.62U/mL GSH reductase. The absorbance was measured at 412 nm and the amount of GSH in each sample was interpolated from a standard curve. For GSSG measurement, supernatants were mixed with 10% triethanolamine and 2-vinylpiridine prior to incubation with the reaction mix.

#### **Statistical analysis**

Results are expressed as mean  $\pm$  standard error of the mean. Differences among the groups were evaluated using the one-way ANOVA test followed by the Tukey test for multiple comparisons. Student's t-test was used to compare between pairs of groups.

#### Electrocardiographic (ECG) telemetry

The protocol for surgical implantation of telemetry devices (PhysioTel Telemetry system, Data Sciences International, St. Paul, MN) to measure biopotential (ECG tracings), temperature, and physical activity in mice has been previously described [9, 53]. Aseptic techniques were used throughout the implantation procedure.

**ECG Data Analysis:** The DataQuest A.R.T. system was used to detect, collect and analyze biopotential, body core temperature and activity telemetry signals from each animal. The acquisition program interfaced with a receiver that was tuned to each animal's implanted ECG telemetry device. At the start of our exposure study, data were sampled each day of exposure for 15 min before, for 5 min every 30 min during the 6 hour exposure period and 15 min post-exposure. As the project progressed, we were able to expand the monitoring to include

monitoring overnight while the animals were housed in the vivarium. The acquisition program automatically cycled through the animals, and acquired data for 5 min out of every 30 min in groups of 4 mice at a time. ECG waveforms were stored on a dedicated computer for subsequent analysis and analyzed to determine heart rate variability (HRV). Changes in HRV may represent alterations in autonomic control of cardiac function [54]. Reduced HRV in humans can represent an adverse effect. Analysis of the ECG waveform was used to extract measures of HRV (the magnitude of variance explained (power) in the heart's rhythm across different frequency bands (spectra) of periodic oscillations in heart rate). Portions of these spectra reflect different autonomic influences on heart rate and blood pressure (BP) [55]. The high frequency (HF) band (1.5 - 5.0 Hz) of the heart period power spectrum has been used to estimate cardiac vagal control [56]. HRV in this band is linked to respiratory influences and has been referred to as "respiratory sinus arrhythmia" [57]. Heart period oscillations at lower frequencies (LF, 0.1 to 1.5 Hz) are less well understood. They may represent mixed sympathetic-parasympathetic and thermoregulatory influences [58-60].

## Results

#### **Concentration and Composition of Ambient PM**

The results of CAPs concentration and composition analyses for the different study locations and years of the exposures are detailed in Table 1. The data shown are means and standard deviations for the CAPs and component concentrations. Ambient  $PM_{2.5}$  concentrations ranged from 7 µg/m<sup>3</sup> to 20 µg/m<sup>3</sup>. The highest ambient concentration was observed in NY but those exposures were performed in 2007. There were differences in the efficiency of operation of the VACES between the 5 locations and at each site there were some day to day variations as well due to fluctuations in ambient temperature and humidity conditions. The highest CAPs concentration was measured at Irvine (138 µg/m<sup>3</sup>) which was approximately twice that measured during the Seattle (61 µg/m<sup>3</sup>) or Michigan (68 µg/m<sup>3</sup>) exposures. This was due to a combination of a slightly greater average ambient PM2.5 concentration and more efficient VACES concentration at UCI as compared with other sites. Elemental Carbon (EC) and Organic Carbon (OC) were measured at all but the Seattle site. EC was higher in NY (1200 ng/m<sup>3</sup>) and Irvine (700 ng/m<sup>3</sup>) than at Sterling Forest (SF; rural) or Michigan (industrial) sites. A similar pattern was seen for OC. The higher concentrations for EC and OC at NY and Irvine could be related to an important influence of vehicular traffic at those sites.

The correlations of elemental constituents with CAPs concentrations at the five exposure locations are summarized in Table 2. Correlations of specific elements that are tracers or surrogates for emissions from specific sources can be used to apportion the influence of specific sources at individual receptor locations. These were excerpted from a more extensive correlation matrix in which each element of the table was correlated with all other elements and from that data one can identify the components that were most strongly associated with the CAPs. Using source apportionment data analysis techniques [61-63], our colleagues at NYU identified some likely candidate elements that could be used to evaluate the possible influence of specific sources to the ambient PM<sub>2.5</sub>.

Some of the emission sources or source types that are likely to contribute to the concentrations of particles in ambient  $PM_{2.5}$  in the five exposure locations are tabulated in Table 3. The specific tracers were identified using source apportionment methods which were described for NY and SF previously [63] and were also applied to the data from Seattle, Michigan and Irvine. The elements selected as tracers provide guidance as to the types of sources influential at each of the sites, but there are significant intercorrelations among these elements so that they are not necessarily unique tracers.

The relative importance of different sources, as indicated by the strength of the correlation of selected source tracer elements, is graphically indicated in Figure 6. Traffic contributions, using EC as a tracer, were important in NYC and UCI exposures. Unfortunately, EC was not measured in SEA. EC is generally associated with the ultrafine size fraction of  $PM_{2.5}$ . The influence of oil combustion using Ni and V as tracers, was highest in NYC but was also substantial for SEA and UCI. Soil and crustal influences using Al and Si as tracers were most important for NYC, SF and SEA. These elements are generally associated with particles 2  $\mu$ m aerodynamic diameter and larger. The impact of wood smoke, using K as a tracer, was greatest in SF and SEA but some influence was also seen in UCI. The findings of an influence of wood smoke at UCI probably reflected incursions of wildfire smoke from uncontrolled burns that occurred in 2010, as opposed to the use of wood burning for heating purposes in SF and SEA.

The impact of coal and industrial emissions using Se as a tracer were greatest in NYC, SF and MSU, all locations with known impacts from coal-burning power plant emissions.

	New York, NY 2007		Sterl. Forest, NY 2007		Seattle, WA 2009		East Lansing, MI 2010		Irvine, CA 2011	
	Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std
Ambient	20	13	17	13	7	4	8	6	10	6
CAPs <sup>2</sup>	123	81	136	111	61	36	68	55	138	89
BC <sup>3</sup>	2667	1521	785	514	1028	755	526	311	1422	1189
EC	1206	765	375	277	$NA^4$	NA	256	241	710	617
OC	8343	6030	3438	2373	NA	NA	5540	1575	7380	1795
AI	1116	623	931	584	858	466	844	539	1006	608
Br	27	19	27	21	20	12	26	16	65	38
Ca	1204	574	223	148	502	290	436	419	690	453
Cr	16	18	19	21	26	19	9	16	17	12
Cu	63	36	22	19	41	28	5	10	87	108
Fe	1879	888	466	362	921	614	302	210	1610	1538
К	429	363	323	300	376	427	298	249	479	215
Mg	360	252	252	201	495	315	291	245	925	648
Mn	107	75	14	12	31	28	16	16	35	32
Na	845	729	502	587	1733	1596	227	287	5024	4540
Ni	70	62	16	16	18	19	7	7	14	12
Р	257	216	219	235	66	68	137	205	142	162
Pb	153	114	55	34	43	23	21	28	25	25
S	11259	10432	17686	19689	3842	2702	6518	7103	7594	5925
Se	9	9	10	12	6	6	20	15	21	13
Si	1658	929	989	798	1399	1005	797	750	1911	1515
TI	77	45	53	34	49	35	21	18	84	74
V	42	44	17	16	26	35	20	13	46	34
Zn	760	1194	78	97	126	90	51	45	100	92
Conc. Fact	or 6.1	5	8.	0	8	3.7	8.:	5		13.8

 Table 1. CAPs Concentrations and Composition Data Averaged For 6 Month Mouse Exposures

<sup>&</sup>lt;sup>2</sup> CAPs concentrations are in  $\mu$ g/m<sup>3</sup>; component concentrations are in ng/m<sup>3</sup>. <sup>3</sup> BC measured using an Aethalometer during the exposure period (0900-1500); EC/OC measured using a Sunset Lab instrument on daily PM collections on quartz filters <sup>4</sup> NA Not Analyzed

	New York, NY (2007)	Sterling Forest, NY (2007)	Seattle, WA (2009)	East Lansing, MI (2010)	Irvine, CA (2011)
CAPs	1	1	1	1	1
BC	0.72	0.45	0.79	0.12	0.44
EC	0.57	0.2	Not Analyzed	0.07	0.34
OC	0.32	0.36	Not Analyzed	-0.19	0.34
AI	0.69	0.7	0.6	0.37	0.2
Br	0.82	0.78	0.65	0.33	0.65
Ca	0.34	0.61	0.54	0.03	0.25
Cr	0.2	-0.08	0.19	-0.16	0.27
Cu	0.47	0.48	0.7	0.11	0.25
Fe	0.34	0.61	0.72	0.39	0.25
К	0.46	0.52	0.62	0.55	0.45
Mg	0.2	0.21	0.14	0.06	0.09
Mn	0.08	0.51	0.56	0.32	0.27
Na	0.2	0.1	0	0.28	0.01
Ni	0.61	0.18	0.35	0.18	0.38
Р	0.59	0.72	0.42	0.68	0.43
Pb	0.26	0.33	0.47	0.04	0.1
S	0.04	0.96	0.76	0.63	0.65
Se	0.47	0.63	0.26	0.38	0.22
Si	0.68	0.63	0.66	0.25	0.22
Ν	0.54	0.67	0.7	0.08	0.29
V	0.64	0.21	0.29	0.03	0.39
Zn	-0.05	0.24	0.51	0.41	0.43

# Table 2. Correlation Coefficient (R) Between Specific Components and CAPs

Table 3. Source Influences on CAPs Composition from Elemental Mass Balance Source Apportionment Methods Based onCorrelations Between Daily Variations in Component Concentrations With Those of PM2.5 Concentrations (Values shown areCorrelation Coefficients; r)

Source	Elemental Indicator	New York, NY (2007)	Sterling Forest, NY (2007)	Seattle, WA (2009)	Michigan State, MI (2010)	Irvine, CA (2011)
Traffic	BC	0.72	0.45	0.79	0.12	0.44
	EC	0.57	0.2	ND	0.07	0.34
Oil	Ni	0.51	0.18	0.35	0.18	0.38
	V	0.64	0.21	0.29	0.03	0.39
Soil	Al	0.69	0.7	0.6	0.37	0.2
	Si	0.58	0.63	0.66	0.25	0.22
Wood Smoke	К	0.46	0.52	0.62	0.55	0.45
Coal	Se	0.47	0.63	0.26	0.38	0.22
Sulfur/Sulfate	S	0.94	0.96	0.76	0.63	0.65



Figure 6. Source Contributions to CAPs Based on a Chemical Mass Balance Tracer Method

#### **Biological Response Data**

#### **New York Sites**

Brain samples from NYC and SF were not provided for our analyses because nearly all the material was used in the aforementioned studies. For comparison with the other three sites, a summary of the results from the New York sites follows. Exposures of mice in New York City (NYC) and Sterling Forest NY (SF) were performed in 2007. Results on biological response analyses from those brains have been summarized by MohanKumar and colleagues in a review article [64]. The brains of CAPs-exposed and FA-exposed apoE-/- mice were multi-embedded and serially sectioned in the coronal plane. The slides were stained for tyrosine hydroxylase (TH), a marker for dopamine (DA) producing neurons, and for glial fibrillary acidic protein (GFAP), a marker of astrocytic proliferation. The nuclei reticularis and compacta of the substantia nigra (SN) were specifically examined because they are anatomical regions containing large populations of DA neurons that are sensitive to oxidative stress and represent the region that is damaged in human and animal models of Parkinson's neuropathy. The SN of each brain was photographed and digitally analyzed. Statistical analysis of the total pixel area of TH stained neurons with their attached fibers showed no significant difference in air or CAPs-exposed normal background control C57/blk6. However, there was a 29% reduction in TH stained neurons in the CAPs exposed brains of apoE-/-mice relative to FA-exposed apoE-/- mice. In addition, there was a significant 8% increase (p<0.05) in GFAP staining (i.e., astrocytes) in the nucleus compacta of CAPs exposed apoE-/- mice relative to FA-exposed apoE-/- mouse brains. While the concentrations of inflammatory cytokines or markers of oxidative stress were not measured in the brains of these mice, samples of the particulate matter collected during the mouse exposures were used in a parallel experiment in which BV2 microglia were incubated with CAPs samples. CAPs stimulated the release of the proinflammatory cytokines tumor necrosis factor-alpha (TNF $\alpha$ ) and interleukin-6 (IL-6) after 6 h of exposures. Microarray analysis of BV2 microglia exposed to high potency CAPs (HP; 75 µg/ml, 4 h) identified 3200 differentially expressed genes (up- and downregulated relative to media controls). The most prominent upregulated gene probes were related to inflammatory pathways associated with Tolllike receptor signaling, MAPK signaling, T- and B-cell receptor signaling, apoptosis, and

elaboration of various proinflammatory cytokines and their receptors. Elemental analysis indicated that the high potency CAPs contained high levels of nickel and vanadium, which may be elemental markers for oil combustion emissions suggesting that particles from sources such as diesel engines and oil-burning power or heating sources can elicit inflammatory responses and can selectively upregulate the expression of inflammatory and innate immunity pathways in BV2 microglia.

#### Irvine, Seattle, and Michigan Sites

#### Brain Regional Differences in Response to Exposure

The present study focused on the remaining three locations, Seattle, WA (SEA), Michigan State University (MSU) and UC Irvine (UCI). The samples from UCI were sectioned into rostral, mid and caudal fractions to examine regional inflammatory and oxidative-stress related responses, how those responses were differentially expressed as a function of length of exposure (2 months vs. 4 months vs. 6 months of exposure), and how those responses progressed from the anterior or front of the brain (rostral) to the posterior or rear of the brain (caudal). Figure 7 summarizes the results for the inflammatory cytokines interleukin-6 (IL-6), interleukin-10 (IL-10) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in cytoplasmic fractions from the three sections of brain tissue. None of the three cytokines demonstrated a consistent trend with time, i.e. average levels across the 3 regions of the brain were not significantly different at 2 months vs. 6 months exposure, although the concentrations for all three cytokines were lower at 4 months than after 2 or 6 months of exposures. The effects of temporal differences in PM concentrations were not analyzed so it is possible that changes in the exposure before a specific measurement period could have influenced the results if the changes were transient. There were however significant regional differences. IL-6 ( $p \le 0.05$ ) and TNF- $\alpha$  ( $p \le 0.10$ ) concentrations were increased in the rostral sections of the brains in CAPs-exposed mice compared to the levels in the FA-exposed mice.

NF $\kappa$ B levels in the nuclear fraction of brain homogenates are shown in Figure 8. There was an increase in NF $\kappa$ B activity in the rostral sections of mice exposed to CAPs at all three time points, which achieved significance after 6 months of exposure (p  $\leq$  0.01). This is consistent with the increased levels of IL-6 and TNF $\alpha$  in rostral sections of the brains of CAPs-exposed

mice, which was depicted in Figure 7. Protein carbonyl concentrations in the cytoplasmic fractions of rostral, midbrain and caudal sections of air-exposed and CAPs-exposed mice are shown in Figure 9. Protein oxidation occurs as a result of reactions of reactive oxygen species (such as superoxide anion) with proteins. Levels of protein carbonyls were significantly higher in the rostral sections in both air- and CAPs-exposed mice however at 2 months of exposure there was a significant increase in rostral sections from CAPs-exposed mice compared with levels in the air-exposed group. After 6 months of exposure, however, the protein carbonyl concentrations in the rostral sections were not significantly different between air-exposed and CAPs-exposed mice but there was a significant (albeit small) increase in the mid-brain sections. It is possible that differences in the exposure in the weeks prior to the measurements could have been related to the variations in protein carbonyl contents but we have not yet examined the data for such trends. It is also possible that some form of adaptation occurred over the course of the study leading to the similarity in values for CAPs-exposed and FA-exposed brain protein carbonyl levels. It is relevant to note that the rostral section has lower NFkB activation levels (Figure 8) than either the midbrain or caudal sections, supporting a role for NFkB in antioxidant defenses of the brain. However, other biomarkers of lipid peroxidation (concentrations of malondialdehyde and hydroxynonenal) were not significantly different across exposure durations, brain sections or exposure conditions (Figure 10).

#### Exposure Site-Specific Differences in Brain Response to CAPs Exposure

The ratio of reduced (GSH) to oxidized (GSSG) glutathione is a sensitive indicator of how exposure can affect the anti-oxidant capacity of cells or tissues. Figure 11 summarizes glutathione levels for homogenized whole brain specimens from the MSU, SEA and UCI exposures. Levels of the active (*i.e.* reduced) glutathione appeared slightly reduced after 6 month exposures at the SEA ( $p \le 0.10$ ) and UCI (n.s.) locations but were not changed in the brains of mice exposed at MSU. Hydroxynonenal levels (Figure 12) were significantly increased in the brains of mice exposed at SEA but there were no differences observed in mice exposed at either MSU or UCI. Exposures of mice to CAPs at SEA ( $p \le 0.05$ ), MSU ( $p \le 0.05$ ) and UCI (n.s.) locations (Figure 13) resulted in lower malondialdehyde (MDA) levels than were observed in the brains of air exposed controls. Both hydroxynonenal and MDA are generally accepted indicators of lipid peroxidation and the finding of lower levels of MDA in the brains of CAPs-

exposed mice while the levels of hydroxynonenal were about the same or slightly increased (as compared with brains from air-exposed mice) is an unusual and at this time unexplained occurrence. Protein carbonyl concentrations (Figure 14) were however increased in the homogenized extract from whole brain samples of mice exposed to CAPs in SEA ( $p \le 0.05$ ) and UCI (not significant) which is consistent with the hydroxynonenal results (Figure 11). It is interesting to note that in UCI samples that were analyzed for just the mid-brain region (Figure 9) there was a significant increase compared to air-exposed mouse brains.

#### **Changes in Cardiac Function and Plaque Development**

The focus of the overall Health Effects Institute (HEI)-funded exposure study was to examine the effects of CAPs exposures on cardiovascular function and atherosclerotic plaque formation. The completed results were submitted as a report to HEI [65]. An important outcome of the NYU study was an appraisal of the progression of plaque development subsequent to exposure. In an independent review of the NYU study by a panel convened by HEI some questions were raised which were articulated in the Executive Summary of the HEI report [65]. Notably, "Surprisingly, few changes were observed at Seattle and Irvine, two major urban areas dominated by traffic-related pollution. ... It remains unclear to what extent the larger responses observed in some locations might have reflected higher CAPs exposures, rather than differences in PM composition. There is also uncertainty in assigning source categories in the factor analyses and it remained unclear why plaque progression in mice exposed to CAPs at Seattle and Irvine was the same as that in mice exposed to filtered air." We examined the plaque progression and compared progression in mice exposed at Irvine compared to those exposed at the Michigan site. The arteries examined were coded as the brachiocephalic (A1), the left common carotid (A2), the left subclavian (A3) and the aortic arch (AA), which are diagrammed in Figure 3. Cerebral arteries were not examined. We found that there was significant plaque development in both FA-exposed and CAPs-exposed mice and that if one examined the data for any single artery the differences between FA and CAPs-exposed groups were small. However as shown in Figure 15, if one computes the number of mice in each group with multiple occluded arteries there is a clear differentiation between the FA and CAPs exposed mice at both the East Lansing and Irvine sites.



Figure 7. Cytokine levels in cytoplasmic fraction of brain homogenates from apoE-/- mice exposed to clean air (blue bars) or fine CAPs (black bars) in Irvine, CA (UCI). n=4 for each exposure group at each time point.



Figure 8. NF-κB levels in nuclear fraction of brain homogenates from apoE-/- mice exposed to clean air (blue bars) or fine CAPs (black bars) in Irvine, CA (UCI). Each bar represents the mean integrated density from a gel shift mobility assay. n=4 for each exposure



Figure 9. Protein carbonyl content in cytoplasmic fraction of brain homogenates from apoE-/- mice exposed to clean air (blue bars) or fine CAPs (black bars) in Irvine, CA (UCI). Each bar represents the mean concentration ± 1 standard error (SE) (n=4) for each exposure time point.





Figure 10. Lipid peroxidation products in cytoplasmic fraction of brain homogenates from apoE-/- mice exposed to clean air (blue bars) or fine CAPs (black bars) in Irvine, CA (UCI). Each bar represents the mean concentration ± 1 standard error (SE) n=4 for each exposure group at each time point. (ND = not detected)



Figure 11. Ratio of Reduced Glutathione (GSH) to oxidized glutathione (GSSG) in brain homogenates from apoE-/- mice exposed for 6 months to clean air (blue bars) or fine CAPs (black bars) at Seattle, WA (SEA), Detroit, MI (MSU) and Irvine, CA (UCI). Values are expressed as mean ± SEM, n=12 per exposure group.



Figure 12. Hydroxynonenal concentrations in cytoplasmic fractions of brain homogenates from apoE-/- mice exposed for 6 months to clean air (blue bars) or fine CAPs (black bars) at Seattle, WA (SEA), Detroit, MI (MSU) and Irvine, CA (UCI). Values are expressed as mean ± SEM, n=12 per exposure group.



Figure 13. Malondialdehyde concentrations in cytoplasmic fractions of brain homogenates from apoE-/- mice exposed for 6 months to clean air (blue bars) or fine CAPs (black bars) at Seattle, WA (SEA), Detroit, MI (MSU) and Irvine, UCI). Values are expressed as mean ± SEM, n=12 for each exposure group.



Figure 14. Protein carbonyl concentrations in cytoplasmic fractions of brain homogenates from apoE-/- mice exposed for 6 months to clean air (blue bars) or fine CAPs (black bars) at Seattle, WA (SEA), Detroit, MI (MSU) and Irvine, UCI). Values are expressed as mean ± SEM, n=12



Figure 15. Number of apoE-/- with Atherosclerotic Lesions in One or More Arteries (brachiocephalic (a1), left common carotid artery (a2), the left subclavian artery (a3) and the aortic arch (AA).

# Conclusions

The analysis of the trace component composition and the source apportionment determinations made using the elemental and compositional tracers provided useful confirmation of the *a priori* criteria used for selecting the exposure locations used in this study. There were differences in exposure concentrations and the efficiency of operation of the VACEs between the five locations. Traffic contributions were important in NYC and UCI exposures. EC, which is a useful tracer for vehicular emission is generally associated with the ultrafine size fraction of PM<sub>2.5</sub>. The influence of oil combustion was highest in NYC but was also substantial for SEA and UCI. NYC has a large base of installed oil-fired power plants within the city limits but most power generation in southern California is from gas-fired facilities. Some of the oil combustion could represent the influence of diesel engines from heavy duty trucks and possibly emissions from the ports at Long Beach and Los Angeles. Soil and crustal influences were most important for NYC, SF and SEA and these sources are generally associated with particles 2 µm aerodynamic diameter and larger. The impact of wood smoke was greatest in SF and SEA but some influence was also seen in UCI. The findings of an influence of wood smoke at UCI might reflect incursions of wildfire smoke from uncontrolled burns that occurred in 2010, as opposed to the use of wood burning for heating purposes in SF and SEA. The impact of coal and industrial emissions were greatest in NYC, SF and MSU, all locations with known impacts from coalburning power plant emissions.

The entire main experiment exposure series took place over a period from 2007 through 2011. The 2007 exposures in NYC and SF were very different from those in SEA, MSU and UCI with respect to concentrations and constituents. In this ARB study we focused on the SEA, MSU and UCI exposure site biological outcome data because the exposures were all performed using mice from the same source, they were done closer in time to each other and the brain sample collections were done in a consistent manner. However, the SEA exposures were performed bracketing the winter months to take advantage of the wood burning activity in Seattle for heating purposes while those in UCI and MSU were performed around the summer period. We had adequate samples to evaluate differences in response in the rostral (front), mid-brain and caudal (rear) regions of the brain for the UCI-exposed mice. However we did note that sectioning

the brain in that manner provided smaller sample masses and increased the analytical variability. We therefore analyzed whole brain homogenates for the SEA and MSU samples.

These differences made it difficult to draw quantitative distinctions between the biological outcomes observed at the various locations. Nevertheless this study showed that CAPs exposures from most of the locations induced biochemical changes in the brains of apoE-/- mice after 6 months of exposure. The largest levels of biomarkers of oxidative stress were seen in the mice exposed to FA and to CAPs at SEA. There were no significant differences between the FA and CAPs-exposed groups at MSU. Relatively consistent increases in biochemical markers of oxidative stress were seen in apoE-/- mice exposed at SEA. The ratio of GSH/GSSG was significantly reduced (CAPs vs. FA) suggesting a decreased antioxidant capacity in the brains. The concentrations of hydroxynonenal and protein carbonyls were significantly increased which when taken together with the GSH/GSSG reduction is convincing evidence that the CAPs exposure induced oxidative stress in these mice. Because the UCI brains had been fractionated, the within group variances in the data for the biomarkers were large when the data were combined to estimate whole brain concentrations that were comparable to the MSU and SEA data sets. Although the differences between FA and CAPs groups were not statistically significant, the directions of the differences were the same as seen for the SEA group.

The UCI data also demonstrated that there were differences in the manner in which different regions of the brain responded to the effects of CAPs exposure. The levels of NF-xB were constitutively different as one progressed from the rostral to the mid-brain to the caudal regions and those levels appeared to decrease as the mice aged from 2 to 4 to 6 months from the start of the exposures (Figure 8). Protein oxidation occurs as a result of reactions of reactive oxygen species (such as superoxide anion) with proteins. Protein carbonyl concentrations in the cytoplasmic fractions of rostral, midbrain and caudal sections of air-exposed and CAPs-exposed mice were significantly higher in the rostral sections in both air- and CAPs-exposed mice; however at 2 months of exposure there was a significant increase in rostral sections from CAPs-exposed mice compared with levels in the air-exposed group (Figure 9). After 6 months of exposure, however, the protein carbonyl concentrations in the rostral sections were not significantly different between air-exposed and CAPs-exposed mice but there was a significant (albeit small) increase in the mid-brain sections. The rostral section has lower concentrations of

activated NF $\kappa$ B than either the midbrain or caudal sections but showed higher concentrations of protein carbonyls than either the midbrain or caudal sections. The finding of significant protein carbonyl increases in regions with lower NF- $\kappa$ B levels could be consistent with a role of NF $\kappa$ B in antioxidant defenses of the brain.

All of the mice in this study were apoE-/- and it has been suggested that these mice are more susceptible to oxidative stress because the apoE protein has antioxidant properties. A study in which protein oxidation in apoE-/- was compared to that in wild type (WT) C57B mice (the strain from which apoE-/- are derived) showed that the total amount of proteins oxidized in the hippocampus of young apoE-/- mice was found to be similar to those of old-WT and old apoE-/- mice, suggesting that there is accelerated oxidative damage due to absence of the apoE gene product [66]. The higher levels of oxidative stress in the FA-exposed mice in this study may have obscured some of the oxidative effects of CAPs exposures on proteins and lipids.

In summary, this study demonstrated that exposure of apoE-/- mice to CAPs was associated with inflammatory changes in the brain, and that on a regional basis, the sections of the brain that were lower in the signal transducer nuclear factor- $\kappa B$  (NF- $\kappa B$ ) tended to be more susceptible to inflammatory changes. In addition the levels of NF-kB decreased as the animals aged during the 6 month study in both Filtered Air (FA) and CAPs-exposed mice. Sections of the brain with lower NF-kB levels also tended to exhibit exposure-related increases in concentrations of biomarkers of oxidative stress, consistent with a correlation between inflammation and oxidative changes in the brain. Oxidative changes in the brain were consistently observed in mice exposed at SEA but not in mice exposed at MSU. Mice exposed at UCI showed a pattern of changes that was the same as that seen in SEA mice but because the UCI brains had been sectioned for brain regional analysis, the variances were larger in the UCI group than in the SEA group and the average effect differences compared to FA-exposed brains did not achieve statistical significance. The sources of PM at the UCI and SEA sites were more influenced by emissions related to oil combustion (such as motor vehicles, power generation, space heating) than was the PM at the MSU site, as evidenced by a high correlation of PM concentrations with the concentrations of Ni and V in the particles at UCI and SEA but not at MSU. The concentrations of Ni and V in the exposure atmosphere were probably too low to be directly toxic and it is likely that these elements are tracers or surrogates for oil combustion aerosols which would include

EC, OC and BC. The pattern of more exposure-related increases in oxidative stress markers at SEA and UCI relative to MSU could indicate that the products of oil combustion from mobile, power generation and space heating sources may be important factors in the inflammatory and oxidative changes noted in the brains of apoE-/- mice exposed to CAPs.

# References

- 1. Lippmann M, Gordon T, Chen LC: Effects of subchronic exposures to concentrated ambient particles in mice: IX. Integral assessment and human health implications of subchronic exposures of mice to CAPs. *Inhalation Toxicology* 2005, 17(4-5):255-261.
- 2. Veronesi B, Makwana O, Pooler M, Chen LC: Effects of subchronic exposures to concentrated ambient particles. VII. Degeneration of dopaminergic neurons in Apo E-/- mice. *Inhalation Toxicology* 2005, 17(4-5):235-241.
- 3. Kreyling WG, Semmler M, Erbe F, Mayer P, Takenaka S, Schulz H, Oberdorster G, Ziesenis A: Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. *Journal of toxicology and environmental health Part A* 2002, 65(20):1513-1530.
- 4. Kreyling WG, Semmler-Behnke M, Seitz J, Scymczak W, Wenk A, Mayer P, Takenaka S, Oberdorster G: Size dependence of the translocation of inhaled iridium and carbon nanoparticle aggregates from the lung of rats to the blood and secondary target organs. *Inhalation Toxicology* 2009, 21 Suppl 1:55-60.
- 5. Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Lunts A, Kreyling W, Cox C: Extrapulmonary translocation of ultrafine carbon particles following whole-body inhalation exposure of rats. *J Toxicol Environ Health A* 2002, 65(20):1531-1543.
- 6. Campbell A, Oldham M, Becaria A, Bondy SC, Meacher D, Sioutas C, Misra C, Mendez LB, Kleinman M: Particulate matter in polluted air may increase biomarkers of inflammation in mouse brain. *Neurotoxicology* 2005, 26(1):133-140.
- 7. Kleinman MT, Araujo JA, Nel A, Sioutas C, Campbell A, Cong PQ, Lia H, Bondy SC: Inhaled ultrafine particulate matter affects CNS inflammatory processes and may act via MAP kinase signaling pathways. *Toxicology Letters* 2008, 178(2):127-130.
- 8. Araujo JA, Barajas B, Kleinman M, Wang X, Bennett BJ, Gong KW, Navab M, Harkema J, Sioutas C, Lusis AJ *et al*: Ambient particulate pollutants in the ultrafine range promote early atherosclerosis and systemic oxidative stress. *Circulation Research* 2008, 102(5):589-596.
- 9. Simkhovich BZ, Kleinman MT, Kloner RA: Particulate air pollution and coronary heart disease. *Current Opinion in Cardiology* 2009, 24(6):604-609.
- 10. Simkhovich BZ, Kleinman MT, Willet P, Gookin G, Salazar K, Keebaugh A, Kloner RA: Chronically inhaled ambient particles cause cardiac inflammation in normal, diseased, and elderly rat hearts. *Air Qual Atmos Hlth* 2011, 4(1):27-36.
- 11. Kleinman MT, Hamade A, Meacher D, Oldham M, Sioutas C, Chakrabarti B, Stram D, Froines JR, Cho AK: Inhalation of concentrated ambient particulate matter near a heavily trafficked road stimulates antigen-induced airway responses in mice. J Air Waste Manage 2005, 55(9):1277-1288.
- 12. Kleinman MT, Sioutas C, Froines JR, Fanning E, Hamade A, Mendez L, Meacher D, Oldham M: Inhalation of concentrated ambient particulate matter near a heavily trafficked road stimulates antigen-induced airway responses in mice. *Inhalation Toxicology* 2007, 19:117-126.

- 13. Meir KS, Leitersdorf E: Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2004, 24(6):1006-1014.
- 14. Lynch JR, Morgan D, Mance J, Matthew WD, Laskowitz DT: Apolipoprotein E modulates glial activation and the endogenous central nervous system inflammatory response. *J Neuroimmunol* 2001, 114(1-2):107-113.
- 15. Zerbinatti CV, Bu G: LRP and Alzheimer's disease. *Rev Neurosci* 2005, 16(2):123-135.
- 16. Ramassamy C, Krzywkowski P, Averill D, Lussier-Cacan S, Theroux L, Christen Y, Davignon J, Poirier J: Impact of apoE deficiency on oxidative insults and antioxidant levels in the brain. *Brain research Molecular brain research* 2001, 86(1-2):76-83.
- 17. Ramassamy C, Averill D, Beffert U, Theroux L, Lussier-Cacan S, Cohn JS, Christen Y, Schoofs A, Davignon J, Poirier J: Oxidative insults are associated with apolipoprotein E genotype in Alzheimer's disease brain. *Neurobiology of disease* 2000, 7(1):23-37.
- 18. Ramassamy C, Averill D, Beffert U, Bastianetto S, Theroux L, Lussier-Cacan S, Cohn JS, Christen Y, Davignon J, Quirion R *et al*: Oxidative damage and protection by antioxidants in the frontal cortex of Alzheimer's disease is related to the apolipoprotein E genotype. *Free Radical Biology & Medicine* 1999, 27(5-6):544-553.
- 19. LaDu MJ, Shah JA, Reardon CA, Getz GS, Bu G, Hu J, Guo L, Van Eldik LJ: Apolipoprotein E and apolipoprotein E receptors modulate A beta-induced glial neuroinflammatory responses. *Neurochemistry international* 2001, 39(5-6):427-434.
- 20. Hafezi-Moghadam A, Thomas KL, Wagner DD: ApoE deficiency leads to a progressive age-dependent blood-brain barrier leakage. *American journal of physiology Cell physiology* 2007, 292(4):C1256-1262.
- 21. Methia N, Andre P, Hafezi-Moghadam A, Economopoulos M, Thomas KL, Wagner DD: ApoE deficiency compromises the blood brain barrier especially after injury. *Mol Med* 2001, 7(12):810-815.
- 22. Grainger DJ, Reckless J, McKilligin E: Apolipoprotein E modulates clearance of apoptotic bodies in vitro and in vivo, resulting in a systemic proinflammatory state in apolipoprotein E-deficient mice. *Journal of Immunology* 2004, 173(10):6366-6375.
- 23. Campbell A, Araujo JA, Li H, Sioutas C, Kleinman M: Particulate matter induced enhancement of inflammatory markers in the brains of apolipoprotein E knockout mice. *Journal of Nanoscience and Nanotechnology* 2009, 9(8):5099-5104.
- 24. Kleinman MT, Araujo JA, Nel A, Sioutas C, Campbell A, Cong PQ, Li H, Bondy SC: Inhaled ultrafine particulate matter affects CNS inflammatory processes and may act via MAP kinase signaling pathways. *Toxicology Letters* 2008, 178(2):127-130.
- 25. Peters A, Veronesi B, Calderon-Garciduenas L, Gehr P, Chen LC, Geiser M, Reed W, Rothen-Rutishauser B, Schurch S, Schulz H: Translocation and potential neurological effects of fine and ultrafine particles a critical update. *Particle and fibre toxicology* 2006, 3:13.
- 26. Oldham MJ, Phalen RF, Robinson RJ, Kleinman MT: Performance of a portable whole-body mouse exposure system. *Inhalation Toxicology* 2004, 16(9):657-662.

- 27. Kim S, Sioutas C, Chang MC, Gong H, Jr.: Factors affecting the stability of the performance of ambient fine-particle concentrators. *Inhalation Toxicology* 2000, 12 Suppl 4:281-298.
- 28. Kleeberger SR, Reddy S, Zhang LY, Jedlicka AE: Genetic susceptibility to ozoneinduced lung hyperpermeability: role of toll-like receptor 4. *Am J Respir Cell Mol Biol* 2000, 22(5):620-627.
- 29. Ohtsuka Y, Clarke RW, Mitzner W, Brunson K, Jakab GJ, Kleeberger SR: Interstrain variation in murine susceptibility to inhaled acid-coated particles. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 2000, 278(3):L469-L476.
- 30. Morokata T, Ishikawa J, Ida K, Yamada T: C57BL/6 mice are more susceptible to antigen-induced pulmonary eosinophilia than BALB/c mice, irrespective of systemic T helper 1/T helper 2 responses. *Immunology* 1999, 98(3):345-351.
- 31. Gee JR, Ding Q, Keller JN: Age-related alterations of Apolipoprotein E and interleukin-1beta in the aging brain. *Biogerontology* 2006, 7(2):69-79.
- 32. Kim S, Jaques PA, Chang MC, Barone T, Xiong C, Friedlander SK, Sioutas C: Versatile aerosol concentration enrichment system (VACES) for simultaneous in vivo and in vitro evaluation of toxic effects of ultrafine, fine and coarse ambient particles - Part II: Field evaluation. *J Aerosol Sci* 2001, 32(11):1299-1314.
- 33. Kim S, Jaques PA, Chang MC, Froines JR, Sioutas C: Versatile aerosol concentration enrichment system (VACES) for simultaneous in vivo and in vitro evaluation of toxic effects of ultrafine, fine and coarse ambient particles Part I: Development and laboratory characterization. *J Aerosol Sci* 2001, 32(11):1281-1297.
- 34. Kim S, Chang MC, Kim D, Sioutas C: A new generation of portable coarse, fine, and ultrafine particle concentrators for use in inhalation toxicology. *Inhalation Toxicology* 2000, 12:121-137.
- 35. Oldham MJ, Phalen RF, Robinson RJ, Kleinman MT: Performance of a portable whole-body mouse exposure system. *Inhal Toxicol* 2004, 16(9):657-662.
- 36. Geller VD, Sardar SB, Phuleria H, Fine PN, Sioutas C: Measurements of particle number and mass concentrations and size distributions in a tunnel environment. *Environmental Science & Technology* 2005, 39(22):8653-8663.
- 37. Ghio AJ, Stonehuerner J, Dailey LA, Carter JD: Metals associated with both the water-soluble and insoluble fractions of an ambient air pollution particle catalyze an oxidative stress. *Inhal Toxicol* 1999, 11(1):37-49.
- **38.** Hlavay J, Polyak K, Weisz M: Monitoring of the natural environment by chemical speciation of elements in aerosol and sediment samples. *J Environ Monit* 2001, 3(1):74-80.
- **39.** Campen MJ, Nolan JP, Schladweiler MC, Kodavanti UP, Evansky PA, Costa DL, Watkinson WP: Cardiovascular and thermoregulatory effects of inhaled PM-associated transition metals: a potential interaction between nickel and vanadium sulfate. *Toxicol Sci* 2001, 64(2):243-252.
- 40. Lippmann M, Ito K, Hwang JS, Maciejczyk P, Chen LC: Cardiovascular effects of nickel in ambient air. *Environmental Health Perspectives* 2006, 114(11):1662-1669.
- 41. Magari SR, Schwartz J, Williams PL, Hauser R, Smith TJ, Christiani DC: The association of particulate air metal concentrations with heart rate variability. *Environ Health Perspect* 2002, 110(9):875-880.

- 42. Natusch DFS, Wallace JR, Evans CA: Toxic Trace-Elements Preferential Concentration in Respirable Particles. *Science* 1974, 183(4121):202-204.
- 43. Carter JD, Ghio AJ, Samet JM, Devlin RB: Cytokine production by human airway epithelial cells after exposure to an air pollution particle is metal-dependent. *Toxicol Appl Pharmacol* 1997, 146(2):180-188.
- 44. Sioutas C, Kim S, Chang M: Development and evaluation of a prototype ultrafine particle concentrator. *J Aerosol Sci* 1999, 30(8):1001-1017.
- 45. Kim S, Sioutas C, Chang MC, Gong H: Factors affecting the stability of the performance of ambient fine-particle concentrators. *Inhalation Toxicology* 2000, 12:281-298.
- 46. Kleinman MT, Hamade A, Meacher D, Oldham M, Sioutas C, Chakrabarti L, Stram D, Froines JR, Cho AK: Inhalation of concentrated ambient particulate matter near a heavily trafficked road stimulates antigen-induced airway responses in mice. *Journal of the Air & Waste Management Association* 2005, 55(9):1277-1288.
- 47. Bolon B, Garman R, Jensen K, Krinke G, Stuart B: A 'best practices' approach to neuropathologic assessment in developmental neurotoxicity testing--for today. *Toxicologic Pathology* 2006, 34(3):296-313.
- 48. Lahiri DK, Ge Y: Electrophoretic mobility shift assay for the detection of specific DNA-protein complex in nuclear extracts from the cultured cells and frozen autopsy human brain tissue. *Brain Res Brain Res Protoc* 2000, 5(3):257-265.
- 49. Campbell A, Becaria A, Lahiri DK, Sharman K, Bondy SC: Chronic exposure to aluminum in drinking water increases inflammatory parameters selectively in the brain. *Journal of neuroscience research* 2004, 75(4):565-572.
- 50. Mohanty JG, Bhamidipaty S, Evans MK, Rifkind JM: A fluorimetric semimicroplate format assay of protein carbonyls in blood plasma. *Analytical biochemistry* 2010, 400(2):289-294.
- 51. Gerard-Monnier D, Erdelmeier I, Regnard K, Moze-Henry N, Yadan JC, Chaudiere J: Reactions of 1-methyl-2-phenylindole with malondialdehyde and 4hydroxyalkenals. Analytical applications to a colorimetric assay of lipid peroxidation. *Chemical Research in Toxicology* 1998, 11(10):1176-1183.
- 52. Griffith OW: Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Analytical biochemistry* 1980, 106(1):207-212.
- 53. Simkhovich BZ, Kleinman MT, Kloner RA: Air pollution and cardiovascular injury epidemiology, toxicology, and mechanisms. *Journal of the American College of Cardiology* 2008, 52(9):719-726.
- 54. Schwartz J, Litonjua A, Suh H, Verrier M, Zanobetti A, Syring M, Nearing B, Verrier R, Stone P, MacCallum G *et al*: Traffic related pollution and heart rate variability in a panel of elderly subjects. *Thorax* 2005, 60(6):455-461.
- 55. Rowan WH, 3rd, Campen MJ, Wichers LB, Watkinson WP: Heart rate variability in rodents: uses and caveats in toxicological studies. *Cardiovascular Toxicology* 2007, 7(1):28-51.
- 56. Pyetan E, Akselrod S: Do the high-frequency indexes of HRV provide a faithful assessment of cardiac vagal tone? A critical theoretical evaluation. *IEEE Trans Biomed Eng* 2003, 50(6):777-783.

- 57. Pyetan E, Akselrod S: A theoretical appraisal of the dependence of respiratory sinus arrhythmia on gradual vagal blockade. *Methods of information in medicine* 2004, 43(1):52-55.
- 58. Shinar Z, Akselrod S, Dagan Y, Baharav A: Autonomic changes during wake-sleep transition: a heart rate variability based approach. *Auton Neurosci* 2006, 130(1-2):17-27.
- 59. Peles E, Goldstein DS, Akselrod S, Nitzan H, Azaria M, Almog S, Dolphin D, Halkin H, Modan M: Interrelationships among measures of autonomic activity and cardiovascular risk factors during orthostasis and the oral glucose tolerance test. *Clin Auton Res* 1995, 5(5):271-278.
- 60. Baharav A, Kotagal S, Gibbons V, Rubin BK, Pratt G, Karin J, Akselrod S: Fluctuations in autonomic nervous activity during sleep displayed by power spectrum analysis of heart rate variability. *Neurology* 1995, 45(6):1183-1187.
- 61. Kleinman MT, Bernstein DM, Kneip TJ: Apparent Effect of Oil Embargo on Total Suspended Particulate Matter and Vanadium in New-York-City Air. *Japca J Air Waste Ma* 1977, 27(1):65-67.
- 62. Kleinman MT, Pasternack BS, Eisenbud M, Kneip TJ: Identifying and Estimating the Relative Importance of Sources of Airborne-Particulates. *Environmental Science* & *Technology* 1980, 14(1):62-65.
- 63. Chen LC, Hwang JS, Lall R, Thurston G, Lippmann M: Alteration of cardiac function in ApoE(-/-) mice by subchronic urban and regional inhalation exposure to concentrated ambient PM2.5. *Inhalation Toxicology* 2010, 22(7):580-592.
- 64. MohanKumar SM, Campbell A, Block M, Veronesi B: Particulate matter, oxidative stress and neurotoxicity. *Neurotoxicology* 2008, 29(3):479-488.
- 65. Lippmann M, Chen LC, Gordon T, Ito K, Thurston GD: National Particle Component Toxicity (NPACT) Initiative: Integrated Epidemiologic and Toxicologic Studies of the Health Effects of Particulate Matter Components. In: *Research Report.* vol. 177. Boston, MA: Health Effects Institute; 2013.
- 66. Choi J, Forster MJ, McDonald SR, Weintraub ST, Carroll CA, Gracy RW: Proteomic identification of specific oxidized proteins in ApoE-knockout mice: relevance to Alzheimer's disease. *Free Radical Biology & Medicine* 2004, 36(9):1155-1162.