Published in final edited form as:

Inhal Toxicol. 2011 August ; 23(0 2): 60–74. doi:10.3109/08958378.2010.563804.

Toxicological Evaluation of Realistic Emission Source Aerosols (TERESA)-power plant studies: assessment of cellular responses

John J. Godleski¹, Edgar A. Diaz¹, Miriam Lemos¹, Mark Long¹, Pablo Ruiz¹, Tarun Gupta¹, Choong-Min Kang¹, and Brent Coull²

¹Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA

²Department of Biostatistics, Harvard School of Public Health, Boston, MA, USA

Abstract

The Toxicological Evaluation of Realistic Emission Source Aerosols (TERESA) project assessed primary and secondary particulate by simulating the chemical reactions that a plume from a source might undergo during atmospheric transport and added other atmospheric constituents that might interact with it. Three coal-fired power plants with different coal and different emission controls were used. Male Sprague-Dawley rats were exposed for 6 h to either filtered air or aged aerosol from the power plant. Four exposure scenarios were studied: primary particles (P); primary + secondary (oxidized) particles (PO); primary + secondary (oxidized) particles + SOA (POS); and primary + secondary (oxidized) particles neutralized + SOA (PONS). Exposure concentrations varied by scenario to a maximum concentration of $257.1 \pm 10.0 \mu\text{g}/\text{m}^3$. Twenty-four hours after exposure, pulmonary cellular responses were assessed by bronchoalveolar lavage (BAL), complete blood count (CBC), and histopathology. Exposure to the PONS and POS scenarios produced significant increases in BAL total cells and macrophage numbers at two plants. The PONS and P scenarios were associated with significant increases in BAL neutrophils and the presence of occasional neutrophils and increased macrophages in the airways and alveoli of exposed animals. Univariate analyses and random forest analyses showed that increases in total cell count and macrophage cell count were significantly associated with neutralized sulfate and several correlated measurements. Increases in neutrophils in BAL were associated with zinc. There were no significant differences in CBC parameters or blood vessel wall thickness by histopathology. The association between neutrophils increases and zinc raises the possibility that metals play a role in this response.

Keywords

Bronchoalveolar lavage; inflammation; histology; inhaled particles; power plant emissions; aerosol sources; pollution sources

Introduction

Pulmonary inflammation is considered an important mechanistic step in the detrimental effects of ambient particle exposures (Li et al., 1997, 1999; Godleski and Clarke 2000; Schins et al., 2004; Cassee et al., 2005; Li et al., 2008). Previous studies from our research

group (Clarke et al., 1999; Clarke et al., 2000a; Clarke et al., 2000b; Saldiva et al., 2002) have used both rat and dog models exposed to concentrated ambient particles (CAPs) to study inflammation as a mechanistic step in understanding the epidemiologic observation that increases in morbidity and mortality are associated with increases in ambient particle pollution (Pope et al., 2004). Our studies have demonstrated that CAPs induce a detectable pulmonary inflammatory reaction in animals using bronchoalveolar lavage (BAL) (Clarke et al., 1999; Clarke et al., 2000b; Saldiva et al., 2002) and confirmed in lung tissue morphometrically (Saldiva et al., 2002). Studies of humans exposed to CAPs also show pulmonary inflammation (Ghio et al., 2000). Autopsy studies on individuals living in areas with high concentration of ambient particles have shown inflammatory alterations of the airways and pulmonary parenchyma, with evidence of particle accumulation within the lungs (Souza et al., 1998).

The primary objective of this study was to evaluate the pulmonary inflammatory potential of primary and secondary particulates derived from coal-fired power plant emissions. By simulating the chemical reactions that a plume from the power plant might undergo during atmospheric transport, realistic complex exposure scenarios were used to test the hypothesis that particles derived from these conditions will produce a pulmonary inflammatory response.

The role of specific sources of particulate pollution in producing an inflammatory response has been a focus of active investigation. Studies have used primary particles from various sources including coal-fired power plants (Gilmour et al., 2004). The role of secondary sulfate particles, primarily derived from SO₂ in power plant emissions, has been considered from both the toxicological and epidemiological viewpoints in recent reviews (Schlesinger, 2007; Reiss et al., 2007). In general, toxicological studies using neutral sulfate show adverse health effects only at very high exposure concentrations. Schlesinger (2007) concluded that inflammation may be due to metals or acid since secondary sulfate particles of public health significance most likely would be those having strong acidity, such as sulfuric acid or ammonium bisulfate, although this acidity is significantly neutralized in the atmosphere as well as in human airways. The relative significance of sulfate-associated H⁺ compared to other PM_{2.5} components is not clear from epidemiology studies that assessed acidity directly (Schlesinger, 2007). In an epidemiological study in the Boston area, Zeka et al. (2006) found associations between particle number, PM_{2.5} mass, and black carbon, but not sulfate, with inflammatory markers. Reiss et al. (2007) concluded that PM sulfate has a weaker “risk factor” than PM_{2.5} for health effects and that since sulfate is correlated with PM_{2.5}, this result is inconsistent with sulfate having a strong health influence. Although both the reviews (Schlesinger, 2007; Reiss et al., 2007) conclude limited health effects from sulfate exposure, both indicate need for more research to delineate the role of specific sources of particulate air pollution.

Ambient particles consist of primary particles emitted from sources of pollution and secondary particles formed by chemical reaction of gases in the atmosphere. Ambient exposures include many sources of air pollution such as power plants, vehicular sources, home heating and combustibles, industrial plants and natural sources. In the Toxicological Evaluation of Realistic Emission Source Aerosols (TERESA) project, health outcomes are studied and related to both primary and secondary particles derived from specific sources of air pollution. In addition, chemical reactions that emissions from the source undergo in a plume during transport from the source to distant sites are simulated through the addition of atmospheric constituents. Health outcomes are then assessed in relationship to the modeled processes as well as measured constituents in the exposure atmospheres.

For these studies, three coal-fired power plants with different sources of coal and with different emissions controls were used as the pollution source. Stack emissions were delivered to a photolytic chamber to simulate, in a compressed period of time, the chemical reactions that occur in the atmosphere, aging this mixture and delivering the fine particulate fraction, along with some gases, to experimental animals. Four different scenarios (experimental exposure models) were used to develop a step-wise increase in complexity of the reactions and simulation of the atmospheric reactions with added atmospheric constituents.

Materials and methods

Power plants

Three coal-fired power plants using different sources of coal and with different emissions controls were included in this study; plant-specific characteristics are presented in detail (Kang et al., 2011). Power plant 1 (PP1) was located in the Upper Midwest, power plant 2 (PP2) located in the Southeast and power plant 3 (PP3) located in the Midwest.

Exposure system

The main features of the exposure facilities have been described in detail (Ruiz et al., 2007a, 2007b; Kang et al., 2011), as have the exposure assessment methods (Kang et al., 2011). Briefly, TERESA involved assessment of actual plant emissions in a field setting using on-site sampling and dilution of coal combustion emissions at three coal-fired power plants. Emissions were introduced into a reaction chamber contained within a mobile laboratory to simulate oxidative atmospheric chemistry, and both primary and secondary products were extensively characterized, including NO₂, SO₂, O₃, NH₃, hydrocarbons, particle mass, particle number, sulfate, nitrate, elemental/organic carbon (EC/OC), ammonium, and elements. The emissions then entered a separate mobile toxicological laboratory where animal exposures were conducted.

Animals

Male Sprague-Dawley CD rats of 250–300 g were obtained from Charles River Laboratories (Portage, MI), delivered directly to the mobile laboratory stationed at the power plant, and housed and managed in a self contained Thoren Maxi-Mizer caging system (Thoren Inc, Hazleton, PA) in the mobile laboratory. Upon arrival, animals were assigned a unique identification number which determined the exposure date and exposure group (aerosol or filtered air) for the animal. Veterinary care and husbandry of the animals was contracted with local universities near each site. These universities also served as overseers of our animal procedures and reported directly to the compliance office for the Harvard Medical Area Standing Committee on the use of animals in research.

Scenarios and experimental design

Four different exposure scenarios were used to evaluate each power plant (Ruiz et al., 2007b): primary particles (P); primary + secondary (oxidized) particles (PO); primary + secondary (oxidized) particles + secondary organic aerosol (SOA) (POS); and primary + secondary (oxidized) particles neutralized + SOA (PONS). At PP3, three control scenarios without primary particles were carried out: oxidized (O), oxidized + SOA (OS), and SOA (S).

Animals were exposed to either an emissions scenario or filtered air by inhalation in individual whole body chambers that also served as plethysmographs for collection of respiratory data (Diaz et al., 2011). For any given scenario, four sets of 6-h exposures were run usually on consecutive days with each set having five animals exposed to the emissions

scenario and five animals exposed to filtered air as controls. Of these, five animals per group, two had *in vivo* chemiluminescence assessments as described in Lemos et al. (2011), and the remaining three were either used for BAL or histopathology as described here. Typically, for animals assessed by BAL, their exposure took place on days 1 and 3 in the sequence, whereas animals assessed by histopathology were exposed on days 2 and 4 in the sequence. The total number of animals used and analyzed for BAL per scenario was 12 (6 aerosol exposed, 6 filtered air controls), and the total number used for histology per scenario was also 12 (6 aerosol exposed, 6 filtered air controls). Assessment of BAL and histopathology was done 24 h after the exposure. Blood for complete blood count (CBC) was collected at the time of sacrifice for the BAL and histopathology so that the total number of animals for this assessment per scenario was 24 (12 aerosol exposed, 12 filtered air controls). A total of 78 exposure days were included in the study. In the introductory paper of this series (Godleski et al., 2011; Rohr et al., 2010), Table 4 of that paper shows the number of animals used in analyses of each outcome at each plant for each scenario.

CBC

Rats were euthanized with an intraperitoneal overdose of barbiturates (Fatal Plus, Vortech Pharmaceuticals Dearborn, Michigan), the thorax was opened, and 2–3 cc of blood was obtained via cardiac puncture for CBC. Blood was collected in 2-ml Vacutainer tubes with EDTA refrigerated, safely packed and shipped via overnight courier service to IDEXX pre-clinical research laboratories in North Grafton, MA, for processing. After collection of blood, the dissection was continued to carry out either BAL assessment or histopathological tissue harvest.

BAL

BAL was carried out using methods previously described (Saldiva et al., 2002). Six consecutive 5 cc washes with endotoxin-free Dulbecco's phosphate-buffered saline were carried out. The first wash was centrifuged and supernatant was collected and frozen with dry ice. Cell resuspension of the first wash plus the other five washes was used for cell count, viability, and cell type determinations. Viability and total cell counts were determined by hemocytometer counts of small aliquots of the resuspended BAL diluted in trypan blue solution. Cell type was determined from modified Wright-Giemsa-stained cytocentrifuge preparations; 200 cells were counted per sample. Supernatant was later used to determine levels of total protein and N-acetyl-beta-glucosaminidase (β -NAG) (Diazyme Corp., San Diego, CA). The former is a marker of pulmonary inflammation and vasculature permeability, whereas the latter is a marker of activation or lysis of phagocytic cells. Total protein was measured using a standard kit from Pierce (Product #23235; Rockford, IL). Determination of β NAG was done by the methods of Sellinger et al. (1960). Enzymatic reagents for the measurement of β NAG were obtained from Sigma Chemical Co (St. Louis, MO), and chemical reagents were obtained from Fisher Scientific Co. (Pittsburgh, PA). β NAG was measured using a kinetic plate reader (Molecular Devices, Sunnyvale, CA).

Histopathology

Preparation of tissues was carried out using methods previously described (Saldiva et al., 2002; Batalha et al., 2002; Lemos et al., 2006). After euthanasia and blood collection, the lungs were fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer. This fixative was delivered to the lung via the trachea at 20 cm of fixative pressure to inflate the lungs, and the pressure was maintained for 30 min. The heart was irrigated with the same fixative solution *in situ* during this period of time. When the heart and lungs were harvested, they were placed in the same 2.5% glutaraldehyde solution in a 100-ml sealed container, and stored at 5°C until further dissection could take place. Total lung volumes were determined by displacement, and the lungs cut horizontally into 2-mm sections which were numbered,

and then three slices were randomly selected for processing by paraffin histology techniques. The heart was cut horizontally into 2-mm sections, numbered, and two slices randomly selected for histological processing by paraffin histology.

Histological approaches used in these studies have also been previously described (Batalha et al., 2002; Saldiva et al., 2002; Lemos et al., 2006). Initially, all slides were assessed qualitatively and descriptively to detect the presence of histopathological alterations of the lung or cardiac parenchyma. In a second step, morphometric methods were applied to subsets of slides to confirm the presence or absence of specific changes in the lungs or heart. For this purpose, the numerical density of the measurement of interest, such as macrophages or neutrophils, (N_i) was determined by counting the number of such cells present in specific locations, at a magnification of 450 $\times$. N_i was assessed using an unbiased counting procedure (Saldiva et al., 2002; Weibel 1986) with the aid of a grid attached to the eyepiece that delineates a square of 225 μm . N_i was corrected by the density of alveolar parenchyma (D_{ap}) in the area of observation, by applying a system of 100 points over the same area where N_i was determined and counting the number of points overlying alveolar tissue. N_i and D_{ap} were determined for 15 microscopic fields for each slide for locations including the centriacinar region (5 fields/slide), defined as those alveolar structures that open directly to airways; and the peripheral acinar region (5 fields/slide), defined as alveoli without an evident relationship with respiratory airways, and large airways (5 fields/slide). Measurements performed for each animal were averaged within each anatomical site to provide a single data point for each location and for each animal. The morphometric measurements were performed by a single observer, with a difference in reproducibility below 10%.

Quantitative measurements of the ratio between the lumen and wall (L/W) areas were done for transverse sections of pulmonary and coronary arteries using the stained slides (Batalha et al., 2002). In the lungs, branches of pulmonary circulation were selected on the basis of their anatomical location, in the transition of airways to alveoli. The second criterion for selection was that the artery in the section needed to be a true cross-section, i.e. having a variation between its maximum and minimum diameter smaller than 10%. For the coronary circulation, cross-sectioned arteries within the outer half of the wall of the left ventricle were evaluated again in true cross-section. In general, at least three arteries fulfilled the criteria in each animal and in each organ. L/W ratios were determined by a standard point counting procedure, at 450 $\times$, with the aid of a coherent system of 100 points contained within a square of 100 by 100 micrometers at this magnification. Briefly, the entire artery was placed within the limits of the square, and the number of points hitting the lumen or the wall of the vessel was determined. An unbiased procedure was adopted, by considering those points lying in the boundary between the lumen and wall as belonging to the lumen of the vessel.

Statistical analyses

The statistical approach used in this paper is described in detail in Coull et al. (2011). Briefly, in a multilayered approach, multiple analyses were conducted that used exposure metrics of increasing sensitivity. First, analysis of variance (ANOVA) techniques that treated exposure as a categorical variable assessed the outcomes applying overall differences between exposed and filtered air responses (i.e. a binary exposure covariate) for each exposure scenario. For all experiments in this paper, we compared response levels in exposed animals to those in the filtered air controls from the same day. For the ANOVA models that assessed the effects of exposure for each scenario, we included a Bonferroni correction for multiple comparisons and considered P -values <0.007 as strong evidence of an exposure effect for a given scenario, and a P -value satisfying $0.007 < P < 0.05$ as marginal evidence of an effect.

In the second level of statistical analyses, univariate associations were assessed between component concentrations and health outcomes. Here, single-component analyses were conducted in which a separate regression model was fitted for each component using differences between exposed and filtered air responses as the outcome and concentration as the exposure metric. The resulting *P*-values from these models were used to rank the strength of associations between each component and health. The third level of analysis, used to support univariate findings, was the concept of variable importance in a random forest analysis used to investigate joint effects of multiple pollution components. An important design issue that impacted the statistical analysis strategy was the fact that the study design exposed animals to both pollution exposures and filtered air across multiple days, which were nested within weeks, which were in turn nested within three power plants. Thus, efficient estimation of health effects required that one identify the levels of this hierarchy that were associated with nuisance variability in each biologic outcome. As a final analysis, adjusted R^2 was used to compare the relative importance of scenario vs. individual components.

Results

Exposure

The exposure concentrations are reported in Table 1 for the three power plants and the four scenarios at each power plant (plus the additional four control scenarios at PP3). Mass exposure concentrations ranged from $1.0 \pm 0.9 \mu\text{g}/\text{m}^3$ for the P scenario at PP1 to $257.1 \pm 10.0 \mu\text{g}/\text{m}^3$ for the PONS scenario at PP2. For mass and sulfate measurements, the concentrations at PP2 were about twice those at PP1, with PP3 having intermediate concentrations between those extremes. The concentration differences between each scenario were proportional within plants. Particle number increased from the P to the PO to the POS, to the PONS scenario at PP1 and PP2, but PP3 had comparatively very high particle numbers in the P, PO, and POS scenarios compared to the other plants. Organic carbon was very low in the P and PO scenarios at all three plants (ranging from 0.0 ± 0.0 to $2.6 \pm 4.5 \mu\text{g}/\text{m}^3$), highest in the POS scenario in all three plants (ranging from 51.6 ± 8.6 to $59.0 \pm 20.5 \mu\text{g}/\text{m}^3$), and also high in the PONS scenario ranging from $30.2 \pm 16.4 \mu\text{g}/\text{m}^3$ in PP1 to $52.0 \pm 23.0 \mu\text{g}/\text{m}^3$ in PP3. This was expected based on the addition of SOA in these two scenarios.

BAL

BAL cellular and fluid parameters are presented in Figures 1 and 2. Figure 1 shows each BAL outcome to each exposure scenario as the difference from filtered air control exposure $\pm$ standard error (SE) for each power plant by scenario. In Figure 2, these data are shown for all plants combined. In considering these two figures together, it is possible to see the contribution of each power plant to a particular outcome (Figure 1) then to view the overall effect of a scenario on that outcome (Figure 2). In Figure 1, total cell counts significantly increased with the POS and PONS scenarios at two of the three plants ($P < 0.001$). This finding persisted when all three plants were considered together (see Figure 2), because individually these BAL outcomes increased at all individual plants even though they reached statistical significance individually at only two. Macrophages significantly increased ($P < 0.001$) in response to the PONS scenario at PP1 and PP3, and when all plants were combined. Total cell number and macrophage numbers for the P and PO scenarios showed little change overall and were not statistically significantly different at any individual plants with these scenarios. Polymorphonuclear neutrophil (PMN) numbers showed a marginally significant increase for the P and PONS scenario at only one of the three plants (PP3); when all plants were combined, the P scenario resulted in higher PMNs. This represents a very modest overall 2.05 ± 1.20 percent change in BAL PMNs, and from Figure 1, it can be seen

that this result is driven solely by PP3. These significant increases at PP3 were sufficient to drive the overall effect even though changes in the P scenario at PP2 were but fractionally increased and at PP2 were essentially 0. Changes in lymphocyte number, protein, and (β -NAG were not significantly different for any scenario at any individual plant, nor overall.

Additional control scenarios were conducted at PP3 as described in the Materials and methods section; none showed any significant changes in BAL parameters (lowest P -value was 0.32, data not shown).

Univariate analyses for total PM mass, particle number, and 21 different measured component concentrations in relationship to BAL total cell count, macrophage number, and PMN number are shown in Table 2. One strongly significant association was observed between PMNs and zinc ($P = 0.0007$). Associations with P -values less than 0.05 for total cell count included increasing concentrations of total sulfate, neutralized sulfate, ammonium ion, and zinc; for macrophage count, associations with P -values less than 0.05 also included total sulfate, neutralized sulfate, and ammonium ion; and for PMNs, a negative association was found with elemental carbon ($P < 0.05$).

Random forest analyses were used in conjunction with univariate analyses results to evaluate component measurements having the greatest influence. Scatter plots on those identified components then show the directionality and the univariate relationship between each change in outcome with increasing concentration of component. The findings of the random forest analysis (Figures 3 and 4) and the scatter plots (Figures 5 and 6) are generally consistent with the scenario and univariate findings. Data for macrophage number and neutrophil number are illustrated. Random forest analyses for macrophage number (Figure 3) found the variables with the strongest influence in this outcome were particle mass, total sulfate, neutral sulfate, and ammonium ion. A clear break is seen between the top 4 and the remainder of the constituents. Figure 5 shows that ammonium ion, neutral sulfate, particle mass, and total sulfate have positive slopes, a distribution across the concentration range, and with POS and PONS scenarios influencing the slopes. Since macrophages dominated the total cell counts, the findings for total cell counts would be expected to be the same. Indeed, for total cell count, the variables with the strongest influence were in order: total sulfate, particle mass, neutral sulfate, and ammonium ion (data not shown). A clear break in the pattern between ammonium ion and the next constituent was observed as with macrophages. For total cells, ammonium ion, neutralized sulfate, mass, and total sulfate all had positive slopes distributed across the concentration range, and with POS and PONS scenarios influencing the slope (data not shown).

Random forest analyses for PMN numbers (Figure 4) found the variable with the strongest influence in this outcome was zinc. Zinc, which was strongest in univariate analysis, is atop the random forest listing, and stands alone. Elemental carbon, which was significant in univariate analyses with a negative coefficient, ranks 6th in the random forest listing. Ozone, which had a nonsignificant negative coefficient in univariate analyses, ranks second but is not close to zinc. The scatter plot for ozone (Figure 6) demonstrates the negative slope. However, the scatter plot for PMN numbers with zinc (Figure 6) shows that these results are driven largely by four high concentration values all at PP3 in the P and PONS scenarios.

In an attempt to determine the relative importance of plant/scenario vs. individual measured component impact on the BAL outcomes, we calculated the adjusted R^2 for plant/scenario ANOVA models and the adjusted R^2 for the component regression models for all outcomes (Table 3) (Coull et al., 2011). For total cell count and total macrophages, plant/scenario models and single-component models explained similar amounts of the variance in the outcomes. For total PMNs, plant/ scenario explained 0.5 which was considerably greater

than other components. The relative strength was plant/ scenario > zinc > mass. Mass explained less than plant/ scenario for all outcomes assessed in Table 3, and less than neutral sulfate, ammonium ion, and total sulfate for macrophages and total cells suggesting that the findings of the POS and PONS scenarios were not simply mass effects.

Histology

On histologic examination, neither increased numbers of macrophages and/or PMNs were observed at bronchoalveolar junctions in any scenario at any plant nor were there any visible pulmonary vascular changes. Both of these were the primary histological findings in our published studies of CAPs (Saldiva et al., 2002; Batalha et al., 2002). Not finding these qualitatively, morphometry was done on at least one scenario from each power plant and confirmed this no effect observation. In Figure 7, the morphological findings in this study are illustrated. Filtered air exposures in all plants with all scenarios showed normal lung and cardiac histology. Figure 7A and 7B shows completely normal alveoli with no evidence of inflammation or cellular increase in filtered air control animals at PP2 and PP3, respectively. Figure 7C shows a representative section from a filtered air control animal at PP3 showing a normal small airway, with adjacent vessels in true cross-section with thin walls. Figure 7D is a section of the heart showing a true cross-section of a coronary artery in a filtered air control animal. There were no histological changes in the heart or coronary arteries in filtered air controls from any power plant or scenario as illustrated in Figure 7D from PP2. There were no histological changes in the heart or in the coronary arteries from TERESA aerosol exposures at any plant or any scenario as illustrated by the example in Figure 7H from PP2, POS scenario.

At PP3, the lung histology of animals exposed to the P and PONS scenarios showed a slight increase in macrophages and rare neutrophils in alveoli. These findings are illustrated in Figure 7E and 7F for the PONS scenario at PP3. Figure 7G illustrates typical findings in the airways of animals exposed to the P and PONS scenario at PP3. An increase in macrophages and rare neutrophils are visible on the surface of the ciliated epithelium of the airways. An increase in parenchymal macrophages or PMNs by morphometry (i.e. macrophages and PMNs at the alveolar level morphologically) was not found with CAPs (Saldiva et al, 2002), and the airway macrophage increase depicted here was also not found with CAPs. On qualitative assessment of macrophages in alveoli and airways at PP1 in all scenarios, there was no difference in numbers of macrophages visible in air spaces or airways as compared to filtered air controls; this finding supports BAL findings in the PO and POS scenarios, but not in the PONS scenario. Limited morphometry on the parenchyma in the PONS scenario at PP1 showed a very slight increase in macrophages which was not statistically significant (controls = 0.10 ± 0.05 ; exposed = 0.12 ± 0.05 ; $P = 0.3$). Animals at PP3 had qualitatively more macrophages in the parenchyma in the PONS scenarios, confirming the BAL findings for that scenario. The histological pictures in Figure 7E, 7F and 7G are representative pictures of the PONS scenario at PP3.

CBC

CBC results for individual plants of all scenarios, as well as combined plant data of all scenarios, are presented in Table 4. TERESA aerosol minus sham differences in CBC parameters, including white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (HGB), hematocrit (HCT), segmented neutrophil count (Neut Seg; a measure of mature neutrophils), band neutrophil count (Neut Band; a measure of immature neutrophils released from the bone marrow as part of an acute inflammatory response), lymphocytes, monocytes, eosinophils, and basophils, are all included. No significant difference in any CBC parameter was found for any scenario at any plant, and also there were no significant differences when data from all plants were combined. As can be seen in Table 4, differences

were extremely small. For example the largest change in WBC count was $1.84 \pm 2.79 \times 10^3$ with the POS scenario at PP2. Throughout the data set, in most instances the standard deviation was larger than the mean difference. In this instance, that observation was not an indication of high variability, rather an indication of very little difference between exposed and control means. Of note, there were no changes in red cell parameters, and there was no increase in band forms of neutrophils in any scenario at any plant.

Discussion

This study was designed to determine whether primary and secondary particles derived from emissions of coal-fired power plants can produce inflammation in the lungs of exposed animals as assessed by BAL and histology, changes in the pulmonary vasculature, and morphological changes in the heart and cardiac vasculature. Results of this study indicate that the POS and PONS scenarios resulted in an increase in total lavaged cells in BAL, and this increase was primarily related to increases in macrophages. Using both univariate and multivariate (random forest) analyses, the BAL increases were most strongly associated with total sulfate, neutral sulfate, and ammonium ion. These are all highly correlated measurements in these experiments; in addition, all are highly correlated with total particle mass, which was also a significant predictor of total cell count in random forest analyses. Increases in PMNs in BAL were found with the P and PONS scenarios at PP3, and statistically related to the concentration of zinc by univariate and random forest analyses. Particle mass concentrations in the P scenario were highest at PP3, likely because of emissions from the wet FGD scrubber used at this plant. Specifically, small amounts of neutral and acidic sulfate were released by the scrubber itself. Of the three plants, P scenario neutral sulfate and particle number concentrations were highest at PP3. The P scenario at PP3 had about 40 times the particle mass concentration compared to the same scenario at the other two plants ($43.2 \pm 14.6 \mu\text{g}/\text{m}^3$ compared to 1.0 ± 0.9 and $1.7 \pm 1.8 \mu\text{g}/\text{m}^3$). However, these observations do not explain the response to the P scenario at PP3 because both total mass and neutral sulfate were much higher in other scenarios at other plants where no responses were observed.

The statistically significant changes in total cell count and macrophages by BAL are considered mild toxicological responses, and these were observed only in scenarios with added SOA and the most complex atmospheric reactions. The SOA used in TERESA was derived from α -pinene, a biogenic volatile organic hydrocarbon. It should be noted that the oxidation products of α -pinene have been studied in a number of toxicological experiments, primarily focused on respiratory responses. Rohr et al. (2002, 2003) reported a number of airway responses due to exposure to α -pinene oxidation products; these included both gas-phase and aerosol-phase products. Pinene-derived SOA has not been examined for any pulmonary inflammatory effects, although the oxidation products of isoprene, another biogenic terpene, have been used in BAL and nasal lavage studies in mice (Rohr, 2001). Isoprene oxidation products, including both gas- and aerosol-phase, showed no evidence of inflammatory activity.

Increases in neutrophils in BAL without increases in BAL protein, enzymes, or circulating neutrophils are also viewed as a mild response. The association with the zinc concentration appears to be driven by increased zinc concentrations in the P and PONS scenarios at PP3 (Figure 6). At PP1 and PP2 for all scenarios, the zinc concentration was very low and ranged from 0.0 to $0.4 \text{ ng}/\text{m}^3$; at PP3, the mean zinc concentrations were 1.5 ± 0.6 for the P scenario, 0.5 ± 0.2 for PO, 1.4 ± 0.9 for POS, and 4.3 ± 2.7 for PONS, which are relatively low, yet all higher than at the other plants (Kang et al., 2011). The role of zinc as a metal in causing health effects has been controversial because all studies showing significant positive inflammatory effects have been done at very high concentration levels (Chen and Lippmann,

2009). Given the distribution of results driving this outcome (Figure 6) and the zinc exposure concentrations in this study (noted above), the role of zinc alone in the etiology of PMN inflammation in this study is not convincing. PP3 had the wet FGD scrubber and the highest mass concentrations in the P scenario among all plants. The contribution of this scrubber to particle generation is discussed in detail by Kang et al. (2011). However, the P and PONS scenarios at PP3 also had substantially higher concentrations of chromium, iron, nickel, as well as zinc compared to these scenarios at PP1 and PP2 (Kang et al., 2011) despite PP1 and PP2 having occasional days with high concentration of these elements. If these concentrations are added together, PP3 had 3–4 times higher concentrations of these elements than the other plants. This difference is likely due to the particular coal burned during the time of the experiments, because the FGD scrubber does not emit metals. The possibility of corrosion associated with the sampling lines is discussed and dismissed as unlikely by Kang et al. (2011). Although the measured concentrations of these metals (other than zinc) did not reach significance in univariate analyses, it is possible that zinc may be a tracer for the combined metal effect.

It is important to compare the results of the studies reported here to CAPs studies in which the same inflammatory endpoints were assessed in order to gain insight into relative response. In CAPs studies in our laboratory in Boston, Clarke et al. (1999) found increases in total lavageable cells with inhalation exposure of normal Sprague-Dawley rats to CAPs as well as neutrophil and lymphocyte counts significantly elevated by CAPs exposure. Saldiva et al. (2002) also found increases in total cells and neutrophils by BAL with CAPs exposure in normal Sprague-Dawley rats exposed for 5 h/day for 3 days, and identified neutrophil increases by morphometry in the bronchiole-alveolar junction region of the lungs. Clarke et al. (2000b) in dog studies in our laboratory also found Boston CAPs to be associated with altered white and red blood cell counts and hemoglobin concentration. These changes with CAPs were all larger in magnitude than the findings in this study. The exposure mass concentration levels in the CAPs studies (Saldiva et al., 2002) were higher, but not appreciably different, to those in this study (average in CAPs rat studies = $255.5 \mu\text{g}/\text{m}^3$; average PONS concentration, all plants = $195.2 \mu\text{g}/\text{m}^3$), suggesting that the CAPs composition, at least in urban Boston, has higher inflammatory potency than the exposures in the present study.

We observed some associations of BAL endpoints with sulfate, which is consistent with some of the literature, including the work of Saldiva et al. (2002) mentioned above, who found increased total BAL cells and neutrophils to be associated with sulfate, among other CAPs components, as well as Clarke et al. (2000b), who found a sulfur factor (determined using factor analytical methods) in CAPs to be associated with altered white and red blood cell counts and hemoglobin concentration. However, other studies have not shown any association with sulfate. Indeed, while a number of CAPs studies in our laboratory and others have shown biological responses (Kodavanti et al., 2000; Gurgueira et al., 2002; Wellenius et al., 2003; Urch et al., 2004; Maciejczyk and Chen, 2005), these studies did not find associations between these effects and either measured sulfate or a sulfate factor. Kodavanti et al. (2000) found that the leachable sulfate in CAPs from Research Triangle Park, NC, was not related to inflammatory responses in rats. Increased activation of nuclear factor- κB in human bronchial epithelial cells did not correlate with sulfate from CAPs from the New York area (where sulfate constituted 65% of the PM mass) (Maciejczyk & Chen, 2005). Another study found that while metals were correlated with the release of cytokines, sulfate was not (Huang et al., 2003). In assessment of cardiovascular outcomes, changes in brachial arterial diameter in human adults were not associated with sulfate in CAPs from Toronto (Urch et al., 2004). Neither increased oxidative stress in rats nor changes in EKG in dogs were associated with a sulfur factor in CAPs from Boston (Gurgueira et al., 2002; Wellenius et al., 2003). Thus, there are also a number of CAPs studies that do not show any

effect of sulfate. It is important to note that CAPs have a very different composition than the aerosols in the TERESA study, and we would not necessarily expect to see the same response. The differences between CAPs in various regions of the United States and TERESA aerosols are discussed in detail in Kang et al. (2011). Moreover, the numbers of animals used in the CAPs studies mentioned above and the numbers of animals used in the studies reported here are similar. It is unlikely that small numbers of animals can explain the lack of robust inflammatory responses in the study reported here.

This scenario, univariate, and multivariate results lacked robust consistency. Scenario-specific results indicate that POS and PONS generally had the largest impact, at least at two of the three plants. Univariate analyses showed that neutral sulfate had one of the stronger associations, while acidic sulfate was uniformly nonsignificant. The POS scenario, which did not contain ammonia, was high in acid sulfate and low in neutral sulfate. Furthermore, the highest neutral sulfate was at PP2 ($139 \mu\text{g}/\text{m}^3$), where no increases in BAL parameters were observed. One hypothesis regarding the acute health effects of sulfate is that the strong acidity sometimes present in this material in the form of sulfuric acid (e.g. Schlesinger, 2007) causes adverse health effects. However, studies with acid sulfate or sulfuric acid have required very high concentrations to elicit biological effects. For example, in human exposure studies with sulfuric acid, concentrations in the range of $1000 \mu\text{g}/\text{m}^3$ were needed to find adverse health effects (Frampton et al., 1992; Linn et al., 1981, 1994). *In vivo* acid neutralization has been used to explain the need for extremely high levels of acid to induce adverse effects (Larson et al., 1980; Sarangapani and Wexler 1996). Concentrations of acid sulfate in our study were highest in the POS scenario at PP2 ($108 \mu\text{g}/\text{m}^3$), averaging $37 \mu\text{g}/\text{m}^3$. In addition, we conducted a control exposure only to acid sulfate ("O" scenario at PP3), which is directly analogous to the aforementioned studies; therefore, our findings are consistent with the lack of effect observed by others. Based on the findings of this study, acid sulfate does not appear to play a significant role in pulmonary inflammatory processes.

Laboratory studies of neutralized sulfates have also been largely negative, or required extremely high concentrations for effects to be observed. In a series of chronic aerosol exposures with acid and neutral sulfur (IV) compound in dogs, no changes in inflammatory parameters were found (Maier et al., 1999), but changes in particle clearance parameters were found (Kreyling et al., 1999). In other toxicological studies of neutralized sulfates, Cassee et al. (2002) exposed normal rats and others to ammonium bisulfate and ammonium ferrosulfate in different particle size ranges for 4 h/day for 3 consecutive days. Animals were sacrificed 1 day after the last exposure. Exposure concentrations were: ultrafine particles = $70 \mu\text{g}/\text{m}^3$; fine particles = $275 \mu\text{g}/\text{m}^3$, $344 \mu\text{g}/\text{m}^3$, or $410 \mu\text{g}/\text{m}^3$. No significant or consistent exposure-related effects were found when assessing some of the same BAL parameters as the study reported here as well as histopathology, and the phagocytic activity to *Escherichia coli* of alveolar macrophages. In another study, Cassee et al. (1998) exposed both normal and ovalbumin sensitized mice for 4 h/day for 3 days to ammonium ferrosulfate ($0.459 \mu\text{m}$) at $250 \mu\text{g}/\text{m}^3$. Animals were sacrificed 1 day after the last exposure, and no significant exposure-related effects on any of the endpoints examined in either group of mice were found, and there was no evidence for any enhanced allergic response due to sulfate. These studies are consistent with the TERESA findings described in this paper. Although we did not study an "ON" scenario, which would directly correspond to the single-component studies described here, we studied scenarios with sulfur in an oxidized form (sulfate) and not containing secondary organic aerosol (i.e. PO). We also did not observe any biological effects with these experimental conditions.

In our study, the addition of secondary organic aerosol appears to be linked with the inflammatory responses observed. Examination of the responses to the control scenarios conducted at PP3 provides valuable information to aid interpretation of the data. As

mentioned, the effects on BAL cellularity in this study were observed primarily with the POS and PONS scenarios. However, the control scenarios, S and OS, both conducted at PP3, showed no significant effects. Organic carbon, the primary indicator for the SOA scenarios, was higher in the control scenarios than in the POS and PONS scenarios at PP3. Therefore, we can conclude that the effect does not appear to be driven by organic material, an observation supported by the lack of significant results for OC in univariate analyses. This might lead to the hypothesis that the primary particles at PP3 are driving the effects on total cells and macrophages; however, there were no significant increases in either BAL parameter for the P scenario at this plant, suggesting that these particles are not playing a role. Other hypotheses would include the formation of organosulfate compounds which could have toxicity in and of themselves; however, previous research to examine this possibility has shown that although organosulfate compounds do form, they are likely not responsible for adverse health impacts (McDonald et al., 2010). Overall, it is unclear why the addition of SOA appears to be important in the BAL responses.

Finally, in comparing results in this study to the respiratory outcomes reported in this series (Diaz et al., 2011) and the chemiluminescence findings in Lemos et al. (2011), there is variable consistency. Peak expiratory flow and expiratory flow at 50% are significantly decreased with the PONS scenario, and that decrease is related to increasing neutral sulfate concentrations among other constituents in univariate analyses, but not in multivariate analyses. Decreases in these expiratory flow parameters are not related to acid sulfate concentration measurements in univariate or multivariate analyses. Indeed, in that paper, increasing acid sulfate concentrations were associated with increasing expiratory flow measurements. Decrements in expiratory flow have been associated with pulmonary inflammatory changes in asthma and chronic obstructive pulmonary disease (Bousquet et al., 2000; Hogg, 2008). Consistent with the increase in total cells and macrophages with the PONS scenario findings of this paper is the observation that lung chemiluminescence is significantly increased in the PONS scenario when data from all plants were combined (Lemos et al., 2011). However, the BAL findings of this study are not consistent with those of Lemos et al. (2011) at the individual plant/scenario level. Marginally significant increased lung chemiluminescence with the POS scenario was found only at PP2 and not at PP1 and PP3 whereas in the present study, total cells and macrophages increased significantly at PP1 and PP3, but no significant effects were observed at PP2. Although lung chemiluminescence increased at all three power plants with the PONS scenario, none of these reached even marginal significance, whereas total cells and macrophages at PP1 and PP3 were significantly increased but PP2 was not. Univariate analyses of lung chemiluminescence and exposure components had no consistent similarities with any BAL parameters and exposure components. Thus, this series of papers, including the findings reported in this paper, must be interpreted as indicating toxicologically mild adverse pulmonary responses to the POS and PONS scenarios which include power plant emissions after reactions with natural gaseous components.

Acknowledgments

The authors thank power plant personnel, the local universities, veterinary clinics, and suppliers who made an extraordinary effort to make a logistically very complex project possible.

Declaration of interest

This project was supported by the Electric Power Research Institute (Contract EP-P10983/C5530/56546), the U.S. Environmental Protection Agency Center, for Particle Health Effects at the Harvard School of Public Health (grant R827353), and the Harvard NIEHS Center for Environmental Health (grant ES00002). This work was also prepared with the support of the U.S. Department of Energy (DOE) under award DE-FC26-03NT41902, and a grant from the State of Wisconsin. However, any opinions, findings, conclusions, or recommendations expressed herein are those of the authors, and do not necessarily reflect the views of the U.S. EPA or the DOE.

References

- Batalha JR, Saldiva PH, Clarke RW, Coull BA, Stearns RC, Lawrence J, Murthy GG, Koutrakis P, Godleski JJ. Concentrated ambient air particles induce vasoconstriction of small pulmonary arteries in rats. *Environ Health Perspect.* 2002; 110:1191–1197. [PubMed: 12460797]
- Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med.* 2000; 161:1720–1745. [PubMed: 10806180]
- Cassee FR, Arts JH, Fokkens PH, Spoor SM, Boere AJ, van Bree L, Dormans JA. Pulmonary effects of ultrafine and fine ammonium salts aerosols in healthy and monocrotaline-treated rats following short-term exposure. *Inhal Toxicol.* 2002; 14:1215–1229. [PubMed: 12454787]
- Cassee FR, Boere AJ, Fokkens PH, Leseman DL, Sioutas C, Kooter IM, Dormans JA. Inhalation of concentrated particulate matter produces pulmonary inflammation and systemic biological effects in compromised rats. *J Toxicol Environ Health Part A.* 2005; 68:773–796. [PubMed: 16020176]
- Cassee, FR.; Dormans, JAMA.; van Loveren, H.; van Bree, L.; Rombout, PJA. Acute toxicity study in asthmatic mice following 3-day exposure to fine ammonium ferrosulfate, a model compound for secondary aerosol fraction of PM10. The Netherlands: Bilthoven; 1998. Toxicity of ambient particulate matter. II.. Report No. 65f0010011.RIVM
- Chen LC, Lippmann M. Effects of metals within ambient air particulate matter (PM) on human health. *Inhalation Toxicology.* 2009; 21:1–31. [PubMed: 18803063]
- Clarke RW, Catalano P, Coull B, Koutrakis P, Krishna Murthy GG, Rice T, Godleski JJ. Age-related responses in rats to concentrated urban air particles (CAPs). *Inhal Toxicol.* 2000a; 12(Supple 1):73–84.
- Clarke RW, Coull B, Reinisch U, Catalano P, Killingsworth CR, Koutrakis P, Kavouras I, Murthy GG, Lawrence J, Lovett E, Wolfson JM, Verrier RL, Godleski JJ. Inhaled concentrated ambient particles are associated with hematologic and bronchoalveolar lavage changes in canines. *Environ Health Perspect.* 2000b; 108:1179–1187. [PubMed: 11133399]
- Clarke RW, Catalano PJ, Koutrakis P, Murthy GG, Sioutas C, Paulauskis J, Coull B, Ferguson S, Godleski JJ. Urban air particulate inhalation alters pulmonary function and induces pulmonary inflammation in a rodent model of chronic bronchitis. *Inhal Toxicol.* 1999; 11:637–656. [PubMed: 10477440]
- Coull BA, Wellenius GA, Gonzalez-Flecha B, Diaz E, Koutrakis P, Godleski JJ. Methods for statistical analysis of TERESA health data. Submitted for publication in this series. 2011; 23(S2): 31–41.
- Diaz EA, Lemos M, Coull BA, Long M, Rohr A, Ruiz P, Gupta T, Kang CM, Godleski JJ. Toxicological Evaluation of Realistic Emissions of Source Aerosols (TERESA): Assessment of Breathing Pattern. Submitted for publication in this series. 2011; 23(S2):42–53.
- Frampton MW, Voter KZ, Morrow PE, Roberts NJ Jr. Culp DJ, Cox C, Utell MJ. Sulfuric acid aerosol exposure in humans assessed by bronchoalveolar lavage. *Am Rev Respir Dis.* 1992; 146:626–632. [PubMed: 1519838]
- Ghio AJ, Kim C, Devlin RB. Concentrated ambient air particles induce mild pulmonary inflammation in healthy human volunteers. *Am J Respir Crit Care Med.* 2000; 162:981–988. [PubMed: 10988117]
- Gilmour MI, O'Connor S, Dick CA, Miller CA, Linak WP. Differential pulmonary inflammation and *in vitro* cytotoxicity of size-fractionated fly ash particles from pulverized coal combustion. *J Air Waste Manag Assoc.* 2004; 54:286–295. [PubMed: 15061611]
- Godleski JJ, Clarke RW. Systemic responses to inhaled ambient particles: Pathophysiologic mechanisms of cardiopulmonary effects. In: *Particle-Lung Interactions* by J., Heyder and P., Gehr eds. Lung Biology in Health and Disease Series-Marcel Dekker, Inc. 2000; 143:577–601.
- Godleski JJ, Rohr AC, Kang CM, Diaz EA, Ruiz PA, Koutrakis P. Toxicological Evaluation of Realistic Emission Source Aerosols (TERESA): Introduction and Overview. *Inhalation Tox.* 2011; 23(S2):95–103.

- Gurgueira SA, Lawrence J, Coull B, Murthy GG, González-Flecha B. Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. *Environ Health Perspect.* 2002; 110:749–755. [PubMed: 12153754]
- Hogg JC. Lung structure and function in COPD. *Int J Tuberc Lung Dis.* 2008; 12:467–479. [PubMed: 18419881]
- Huang SL, Hsu MK, Chan CC. Effects of submicrometer particle compositions on cytokine production and lipid peroxidation of human bronchial epithelial cells. *Environ Health Perspect.* 2003; 111:478–482. [PubMed: 12676602]
- Kang CM, Gupta T, Ruiz PA, Wolfson JM, Ferguson ST, Lawrence J, Rohr AC, Godleski JJ, Koutrakis P. Aged particles derived from emissions of coal-fired power plants: The TERESA field results. *Inhalation Toxic.* 2011; 23(S2):11–30.
- Kodavanti UP, Mebane R, Ledbetter A, Krantz T, McGee J, Jackson MC, Walsh L, Hilliard H, Chen BY, Richards J, Costa DL. Variable pulmonary responses from exposure to concentrated ambient air particles in a rat model of bronchitis. *Toxicol Sci.* 2000; 54:441–451. [PubMed: 10774827]
- Kreyling WG, Dirscherl P, Ferron GA, Heilmann P, Josten M, Miaskowski U, Neuner M, Reitmeir P, Ruprecht L, Schumann G, Takenaka S, Ziesenis A, Heyder J. Health effects of sulfur-related environmental air pollution. III. Nonspecific respiratory defense capacities. *Inhal Toxicol.* 1999; 11:391–422. [PubMed: 10380176]
- Larson T, Frank R, Covert D, Holub D, Morgan M. The chemical neutralization of inhaled sulfuric acid aerosol. *Am J Ind Med.* 1980; 1:449–452. [PubMed: 7342781]
- Lemos M, Diaz E, Gupta T, Kang C-M, Ruiz P, Coull B, Gonzalez-Flecha B. Cardiac and pulmonary oxidative stress in rats exposed to realistic emissions of source aerosols. Submitted for publication in this series. 2011; 23(S2):75–83.
- Lemos M, Mohallem S, Macchione M, Dolhnikoff M, Assunção JV, Godleski JJ, Saldiva PNH. Chronic Exposure to Urban Air Pollution Induces Structural Alterations in Murine Pulmonary and Coronary Arteries. *Inhalat Toxic.* 2006; 18:247–253.
- Li XY, Brown D, Smith S, MacNee W, Donaldson K. Short-term inflammatory responses following intratracheal instillation of fine and ultrafine carbon black in rats. *Inhal Toxicol.* 1999; 11:709–731. [PubMed: 10477444]
- Li XY, Gilmour PS, Donaldson K, MacNee W. *In vivo* and *in vitro* proinflammatory effects of particulate air pollution (PM10). *Environ Health Perspect.* 1997; 105(Suppl 5):1279–1283. [PubMed: 9400738]
- Linn WS, Kleinman MT, Bailey RM, Medway DA, Spier CE, Whynot JD, Anderson KR, Hackney JD. Human respiratory responses to an aerosol containing zinc ammonium sulfate. *Environ Res.* 1981; 25:404–414. [PubMed: 7274202]
- Linn WS, Shamoo DA, Anderson KR, Peng RC, Avol EL, Hackney JD. Effects of prolonged, repeated exposure to ozone, sulfuric acid, and their combination in healthy and asthmatic volunteers. *Am J Respir Crit Care Med.* 1994; 150:431–440. [PubMed: 8049826]
- Maier KL, Beck-Speier I, Dayal N, Dirscherl P, Griese M, Heilmann P, Hinze H, Josten M, Karg E, Kreyling WG, Lenz A, Leuschel L, Meyer B, Miaskowski U, Reitmeir P, Ruprecht L, Schumann G, Ziesenis A, Heyder J. Health effects of sulfur-related environmental air pollution. II. Cellular and molecular parameters of injury. *Inhal Toxicol.* 1999; 11:361–389. [PubMed: 10380175]
- Maciejczyk P, Chen LC. Effects of subchronic exposures to concentrated ambient particles (CAPs) in mice: VIII. Source-related daily variations in *in vitro* responses to CAPs. *Inhal Toxicol.* 2005; 17:243–253. [PubMed: 15804942]
- McDonald JD, Doyle-Eisele M, Campen MJ, Seagrave J, Holmes T, Lund A, Surratt JD, Seinfeld JH, Rohr AC, Knipping EM. Cardiopulmonary response to inhalation of biogenic secondary organic aerosol. *Inhal Toxicol.* 2010; 22:253–265. [PubMed: 20148748]
- Pope CA 3rd, Burnett RT, Thurston GD, Thun MJ, Calle EE, Krewski D, Godleski JJ. Cardiovascular mortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. *Circulation.* 2004; 109:71–77. [PubMed: 14676145]
- Reiss R, Anderson EL, Cross CE, Hidy G, Hoel D, McClellan R, Moolgavkar S. Evidence of health impacts of sulfate- and nitrate-containing particles in ambient air. *Inhal Toxicol.* 2007; 19:419–449. [PubMed: 17365047]

- Rohr, AC. Doctor of Science Dissertation. Boston, MA: Harvard University School of Public Health; 2001. Effects of terpene/ozone reaction products on the murine respiratory system.
- Rohr AC, Rohr AC, Shore SA, Spengler JD. Repeated exposure to terpene oxidation products causes enhanced respiratory tract effects in multiple murine strains. *Inhal Toxicol.* 2003; 15:1191–1197. [PubMed: 14515222]
- Rohr AC, Wilkins CK, Clausen PA, Hammer M, Nielsen GD, Spengler JD, Wolkoff P. Upper airway and pulmonary effects of oxidation products of (+)- α -pinene, d-limonene, and isoprene in BALB/c mice. *Inhal Toxicol.* 2002; 14:663–684. [PubMed: 12122569]
- Ruiz PA, Lawrence JE, Wolfson JM, Ferguson ST, Gupta T, Kang CM, Koutrakis P. Development and evaluation of a photochemical chamber to examine the toxicity of coal-fired power plant emissions. *Inhal Toxicol.* 2007a; 19:597–606. [PubMed: 17510833]
- Ruiz PA, Gupta T, Kang CM, Lawrence JE, Ferguson ST, Wolfson JM, Rohr AC, Koutrakis P. Development of an exposure system for the toxicological evaluation of particles derived from coal-fired power plants. *Inhal Toxicol.* 2007b; 19:607–619. [PubMed: 17510834]
- Saldiva PHN, Clarke RW, Coull BA, Stearns R, Lawrence J, Koutrakis P, Suh H, Tsuda A, Godleski II. Acute pulmonary inflammation induced by concentrated ambient air particles is related to particle composition. *Amer J Respir Crit Care Med.* 2002; 165:1610–1617. [PubMed: 12070061]
- Sarangapani R, Wexler AS. Growth and neutralization of sulfate aerosols in human airways. *J Appl Physiol.* 1996; 81:480–490.
- Schlesinger RB. The health impact of common inorganic components of fine particulate matter (PM_{2.5}) in ambient air: a critical review. *Inhal Toxicol.* 2007; 19:811–832. [PubMed: 17687714]
- Schins RP, Lightbody JH, Borm PJ, Shi T, Donaldson K, Stone V. Inflammatory effects of coarse and fine particulate matter in relation to chemical and biological constituents. *Toxicol Appl Pharmacol.* 2004; 195:1–11. [PubMed: 14962500]
- Sellinger OZ, Beaufay H, Jacques P, Doyen A, De DUVE C. Tissue fractionation studies. 15. Intracellular distribution and properties of beta-N-acetylglucosaminidase and beta-galactosidase in rat liver. *Biochem J.* 1960; 74:450–456. [PubMed: 14444755]
- Souza MB, Saldiva PH, Pope CA3rd, Capelozzi VL. Respiratory changes due to long-term exposure to urban levels of air pollution: a histopathologic study in humans. *Chest.* 1998; 113:1312–1318. [PubMed: 9596312]
- Urch B, Brook JR, Wasserstein D, Brook RD, Rajagopalan S, Corey P, Silverman F. Relative contributions of PM_{2.5} chemical constituents to acute arterial vasoconstriction in humans. *Inhal Toxicol.* 2004; 16:345–352. [PubMed: 15204750]
- Weibel, ER. Functional morphology of lung parenchyma. In: Fishman, AP., editor. *Handbook of physiology: The respiratory system*. Bethesda: American Physiological Society; 1986. p. 89–111.
- Wellenius GA, Coull BA, Godleski II, Koutrakis P, Okabe K, Savage ST, Lawrence JE, Murthy GG, Verrier RL. Inhalation of concentrated ambient air particles exacerbates myocardial ischemia in conscious dogs. *Environ Health Perspect.* 2003; 111:402–408. [PubMed: 12676590]
- Zeka A, Sullivan JR, Vokonas PS, Sparrow D, Schwartz I. Inflammatory markers and particulate air pollution: characterizing the pathway to disease. *Int J Epidemiol.* 2006; 35:1347–1354. [PubMed: 16844771]

Figure 1.

Differences in BAL parameters by scenario and power plant: Increases in total cells and macrophages are seen with the POS and PONS scenarios in all power plants, and are significantly increased in two of three plants. The P and PO scenarios have small insignificant changes in PP1 and PP2. Significantly increased PMNs are found in PP3 in the P and PONS scenarios. Other parameters have minimal variable changes.

Aerosol vs. Sham Differences in BAL
 $\Delta \pm \text{SE}$ by Parameter, Scenario, For All Power Plants

Figure 2.

Differences in BAL parameters by scenario: Data from all power plants combined show the difference between filtered air control and TERESA aerosol exposed animals by scenario in all power plants combined. The POS and PONS scenarios show significant increases in total BAL cells and macrophage numbers. The P scenario has a significant increase in PMNs.

Random Forest Variable Importance—Macrophage Count

Figure 3. Random forest ranking the effect of exposure components in change of BAL total macrophage count: top ranked and separated components are mass, total sulfate, neutralized S04, and ammonium ion.

Random Forest Variable Importance—PMN Count

Figure 4. Random forest ranking the effect of exposure components in change of BAL PMN count: top ranked and most separated component is zinc.

Figure 5.

Scatter plots for BAL macrophage count: each point has a color and symbol indicating specific plants and scenarios. Mass, total sulfate, neutral sulfate, and ammonium ion all show positive associations. Total sulfate has a uniform distribution with the POS and PONS scenarios making mostly positive contributions.

Figure 6.

Scatter plots for BAL PMN count: each point has a color and symbol indicating specific plants and scenarios. The zinc result appears to be driven by a small number of high concentrations at PP3 in the PONS and P scenarios. PMN count is negatively associated with ozone. Mass has no association, and neutral sulfate has a slight positive slope, but univariate and random forest analyses indicate no significant associations.

Figure 7. Histopathology of lungs and heart in filtered air exposed control animals (A–D) and PONS aerosol exposed animals from PP3 (E–H). No pathological changes are visible in the control animal parenchyma, airways, pulmonary vessels, myocardium or cardiac vessels. In the exposed animals, increases in alveolar macrophages and rare neutrophils are visible in alveoli. The airway in panel G has increased macrophages on the epithelial surface. There are no changes in the myocardium or cardiac vessels in panel H. Bar = 200 μm in panel A, all other original magnifications are 400×.

Table 1
Summary of aged particulate species derived from three coal-fired power plants for the TERESA study.

Power plant	Exposure scenario	Aged particle mass (µg/m)	Particle number (#/cm ³)	Total sulfate (µg/m)	Acidic sulfate ¹ (µg/m)	Neutralized sulfate ² (µg/m)	Organic carbon (µg/m)
Plant 1	P(n = 4) ³	1.0±0.9	1726±1277	0.2 ±0.3	2.3 ±0.4	0.0±0.0	0.0±0.0
	pO(n = 3)	46.0±12.6	6723±3550	36.1±7.7	27.6±9.5	8.4±2.6	2.6±4.5
	pOS(n = 4)	123.3±28.4	16924±4495	55.8±22.8	50.2±21.6	5.6±3.4	51.6±8.6
	PONS (n = 12)	154.9±41.7	52109±11951	68.2±28.8	14.7 ±13.6	53.6±16.8	30.2 ±16.4
Plant 2	P(n = 4)	1.7±1.8	910±964	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	pO(n = 4)	115.5±18.5	4281±1911	100.3 ±16.3	71.6±17.0	28.8 ±1.3	0.0±0.0
	POS(n = 8)	212.1±39.7	11473±3774	146.0±36.7	107.9±31.7	38.1 ±12.0	59.0 ±20.2
	PONS(n = 4)	257.1±10.0	40811±2179	154.8 ±12.4	15.7±3.8	139.1 ±15.5	35.1 ±10.1
Plant 3	P(n = 4)	43.2 ±14.6	55947±11769	34.0 ±13.3	12.8±7.1	21.2±9.2	1.9±3.8
	pO(n = 4)	82.3±15.6	69372±8523	77.9 ±14.5	66.6±16.8	10.3±2.8	0.0±0.0
	POS(n = 8)	144.4±31.6	40446±6657	83.3±21.3	68.9±18.2	14.5±7.1	54.7±27.5
	PONS(n = 4)	173.5±20.9	38483±3651	85.0 ±12.9	2.5±2.0	82.5 ±13.5	52.0±23.0
	OS(n = 4)	137.8 ±9.3	35959±6290	47.2 ±14.6	30.3±11.6	16.9±11.6	83.6 ±9.6
	O(n = 4)	43.8±3.5	29294±2392	40.6±3.8	31.7±5.8	8.9±2.3	0.0±0.0
	S(n = 4)	61.4±6.6	7574±1598	1.3 ±0.4	1.0±1.3	0.7±0.5	59.7±6.1

¹ Acidic sulfate was calculated from strong acidity (pH) measurements as the equivalent concentration of H₂SO₄ aerosol;

² Neutralized sulfate = Total sulfate - Acidic sulfate;

³ Number of days; All values are average ± standard deviation.

Table 2
Univariate analysis standardized BAL parameter changes vs. aerosol composition.

	Total cell count		Macrophages		PMN	
	Coef±SE(×10 ³)	P-value	Coef±SE(×10 ³)	P-value	Coef±SE(×10 ³)	P-value
Mass	727.21±509.65	0.15	670.98±465.76	0.15	-8.04±35.96	0.82
PN	-159.02±525.34	0.76	-105.77±480.72	0.83	51.35±34.79	0.14
Total SO4	1131.64±485.27	0.02	1005.50±445.91	0.02	4.22 ± 35.99	0.91
Acidic SO4	372.84±536.29	0.49	243.13±490.83	0.62	-41.23±36.28	0.26
Neutralized SO4	1117.68±500.60	0.03	1079.18±451.73	0.02	42.28±36.24	0.24
NH4	1066.75±489.99	0.03	1036.90±443.59	0.02	33.95±35.47	0.34
OC	-234.46 ±524.43	0.65	-229.06±479.33	0.63	-17.45±35.85	0.63
EC	103.82±525.79	0.84	47.57±481.02	0.92	-70.25±33.71	0.04
TC	-207.11±524.80	0.69	-209.53±479.62	0.66	-25.74±35.69	0.47
Al	164.22±495.60	0.74	-57.76±457.53	0.90	57.07±35.61	0.11
Si	-682.77±480.60	0.16	-548.78±446.55	0.22	-11.08±37.05	0.76
Fe	564.95±485.67	0.24	413.61±451.38	0.36	51.08±35.91	0.15
Ni	571.60±485.41	0.24	447.65±450.29	0.32	29.77±36.71	0.42
Zn	977.71±457.44	0.03	670.52±439.44	0.13	109.06±32.01	0.0007
Pb	195.05±495.22	0.69	219.72±455.89	0.63	-20.44±36.92	0.58
Na	601.76±484.19	0.21	390.82±452.05	0.39	57.63±35.58	0.11
O3	761.77±508.02	0.13	563.73±470.32	0.23	-36.38±35.39	0.30
NO	-493.94±518.59	0.34	-555.43±470.64	0.24	9.89±35.95	0.78
NO2	708.33±510.51	0.17	773.76±460.58	0.09	-1.37±35.99	0.97
NO3	239.58±524.36	0.65	379.38±476.24	0.43	-5.33±35.98	0.88
SO2	-62.03±526.00	0.91	-360.00±476.73	0.45	-21.90±35.77	0.54
Phene	-392.63±521.37	0.45	-263.08±478.77	0.58	-2.26±35.98	0.95

Shaded = $P < 0.05$, Coef= Change in A per SD of the concentration.

Table 3

Comparison of adjusted R^2 for plant/scenario and various exposure metrics identified in univariate and random forest analyses.

Parameter	Scenario /plant	Mass	Neutral sulfate	Ammonium ion	Total sulfate	Zinc	Elemental carbon
Total cell count	-0.074	0.031	0.114	0.105	0.122	0.25	-0.031
Total macrophages	-0.155	0.033	0.132	0.122	0.113	0.07	-0.032
Total PMNs	0.514	-0.031	0.011	-0.003	-0.032	0.13	0.095

Table 4
Aerosol vs. sham differences in CBC parameters A $\pm$ standard deviation by parameter, scenario and power plant.

Parameter	WBC		RBC		HGB		HCT		Neut seg		Neut band		Lymphocyte		Monocyte		Eosinophils		Basophiles	
	A $\pm$ SD		A $\pm$ SD		A $\pm$ SD		A $\pm$ SD		A $\pm$ SD		A $\pm$ SD		A $\pm$ SD		A $\pm$ SD		A $\pm$ SD		A $\pm$ SD	
Power plant 1	PO	0.08 $\pm$ 1.35	-0.08 $\pm$ 0.60	0.26 $\pm$ 0.99	0.52 $\pm$ 3.97	451 $\pm$ 418	0 $\pm$ 0	-325 $\pm$ 1217	-28 $\pm$ 161	-16 $\pm$ 36	0 $\pm$ 0									
	POS	-1.10 $\pm$ 4.43	0.12 $\pm$ 0.57	0.13 $\pm$ 0.99	-0.83 $\pm$ 3.15	-338 $\pm$ 392	0 $\pm$ 0	-708 $\pm$ 1965	-88 $\pm$ 151	-10 $\pm$ 30	0 $\pm$ 0									
	PONS	-0.25 $\pm$ 3.44	-0.05 $\pm$ 0.42	-0.11 $\pm$ 0.61	-0.22 $\pm$ 2.35	74 $\pm$ 600	0 $\pm$ 0	-198 $\pm$ 2864	50 $\pm$ 136	10 $\pm$ 62	-4 $\pm$ 15									
Power plant 2	P	-1.68 $\pm$ 1.88	0.29 $\pm$ 0.41	0.27 $\pm$ 0.84	-0.22 $\pm$ 2.79	-327 $\pm$ 876	0 $\pm$ 0	-1402 $\pm$ 1551	-29 $\pm$ 108	-2 $\pm$ 34	0 $\pm$ 0									
	PO	0.23 $\pm$ 3.60	0.26 $\pm$ 0.87	0.28 $\pm$ 0.88	1.01 $\pm$ 6.11	430 $\pm$ 529	0 $\pm$ 0	-136 $\pm$ 3070	-27 $\pm$ 238	-40 $\pm$ 103	-12 $\pm$ 35									
	POS	1.84 $\pm$ 2.79	0.30 $\pm$ 0.28	0.39 $\pm$ 0.46	1.27 $\pm$ 2.08	152 $\pm$ 527	0 $\pm$ 0	2008 $\pm$ 1985	-69 $\pm$ 644	22 $\pm$ 38	0 $\pm$ 0									
Power plant 3	PONS	-1.27 $\pm$ 4.14	0.31 $\pm$ 0.37	0.80 $\pm$ 0.44	1.87 $\pm$ 1.45	-350 $\pm$ 979	0 $\pm$ 0	-905 $\pm$ 3070	-5 $\pm$ 300	-7 $\pm$ 39	0 $\pm$ 0									
	P	-0.74 $\pm$ 2.51	0.02 $\pm$ 0.34	0.02 $\pm$ 0.47	-0.36 $\pm$ 1.74	-246 $\pm$ -246	0 $\pm$ 0	-407 $\pm$ 1856	-86 $\pm$ 130	4 $\pm$ 39	0 $\pm$ 0									
	PO	0.26 $\pm$ 2.29	-0.07 $\pm$ 0.59	-0.02 $\pm$ 1.06	-0.32 $\pm$ 3.01	90 $\pm$ 90	0 $\pm$ 0	145 $\pm$ 2043	52 $\pm$ 161	-29 $\pm$ 98	0 $\pm$ 0									
All plants combined	POS	1.42 $\pm$ 2.24	0.04 $\pm$ 0.36	-0.01 $\pm$ 0.58	0.83 $\pm$ 3.09	440 $\pm$ 440	0 $\pm$ 0	1012 $\pm$ 2390	-7 $\pm$ 76	52 $\pm$ 43	0 $\pm$ 0									
	PONS	0.28 $\pm$ 1.26	0.41 $\pm$ 1.16	0.61 $\pm$ 2.14	-2.83 $\pm$ 3.12	69 $\pm$ 69	0 $\pm$ 0	935 $\pm$ 616	-16 $\pm$ 16	-2 $\pm$ 17	0 $\pm$ 0									
	P	-1.05 $\pm$ 2.31	0.11 $\pm$ 0.37	0.10 $\pm$ 0.61	-0.31 $\pm$ 2.06	-273 $\pm$ 876	0 $\pm$ 0	-739 $\pm$ 1780	-67 $\pm$ 123	2 $\pm$ 37	0 $\pm$ 0									
All plants combined	PO	0.21 $\pm$ 2.57	0.04 $\pm$ 0.69	0.15 $\pm$ 0.96	0.34 $\pm$ 4.39	294 $\pm$ 497	0 $\pm$ 0	-65 $\pm$ 2241	8 $\pm$ 184	-28 $\pm$ 85	0 $\pm$ 0									
	POS	0.78 $\pm$ 3.48	0.19 $\pm$ 0.41	0.22 $\pm$ 0.69	0.54 $\pm$ 2.76	103 $\pm$ 695	0 $\pm$ 0	1105 $\pm$ 2313	-59 $\pm$ 457	22 $\pm$ 46	0 $\pm$ 0									
	PONS	-0.25 $\pm$ 3.03	0.17 $\pm$ 0.74	0.25 $\pm$ 1.31	-0.24 $\pm$ 2.66	-2 $\pm$ 646	0 $\pm$ 0	-89 $\pm$ 2611	26 $\pm$ 161	5 $\pm$ 52	0 $\pm$ 0									