Inhaled Concentrated Ambient Particles Are Associated with Hematologic and Bronchoalveolar Lavage Changes in Canines

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Pulmonary inflammatory and hematologic responses of canines were studied after exposure to concentrated ambient particles (CAPs) using the Harvard ambient particle concentrator (HAPC). For pulmonary inflammatory studies, normal dogs were exposed in pairs to either CAPs or filtered air (paired studies) for 6 hr/day on 3 consecutive days. For hematologic studies, dogs were exposed for 6 hr/day for 3 consecutive days with one receiving CAPs while the other was simultaneously exposed to filtered air; crossover of exposure took place the following week (crossover studies). Physicochemical characterization of CAPs exposure samples included measurements of particle mass, size distribution, and composition. No statistical differences in biologic responses were found when all CAPs and all sham exposures were compared. However, the variability in biologic response was considerably higher with CAPs exposure. Subsequent exploratory graphical analyses and mixed linear regression analyses suggested associations between CAPs constituents and biologic responses. Factor analysis was applied to the compositional data from paired and crossover experiments to determine elements consistently associated with each other in CAPs samples. In paired experiments, four factors were identified; in crossover studies, a total of six factors were observed. Bronchoalveolar lavage (BAL) and hematologic data were regressed on the factor scores. Increased BAL neutrophil percentage, total peripheral white blood cell (WBC) counts, circulating neutrophils, and circulating lymphocytes were associated with increases in the aluminum/silicon factor. Increased circulating neutrophils and increased BAL macrophages were associated with the vanadium/nickel factor. Increased BAL neutrophils were associated with the bromine/lead factor when only the compositional data from the third day of CAPs exposure were used. Significant decreases in red blood cell counts and hemoglobin levels were correlated with the sulfur factor. BAL or hematologic parameters were not associated with increases in total CAPs mass concentration. These data suggest that CAPs inhalation is associated with subtle alterations in pulmonary and systemic cell profiles, and specific components of CAPs may be responsible for these biologic responses. *Key words*: bronchoalveolar lavage, canines, environmental particles, hematology, inflammation. *Environ Health Perspect* 108:1179–1187 (2000). [Online 15 November2000] *http://ehpnet1.niehs.nih.gov/docs/2000/108p1179-1187clarke/abstract.html*

Epidemiologic studies have associated human morbidity and mortality with exposure to ambient air particles (*1–4*). Toxicologic responses to particle exposures include acute mortality (*5*), histopathologic changes in the airways (*6,7*), increased airway hyperresponsiveness (*7–9*), pulmonary inflammation (*10–15*), and depressed pulmonary defense mechanisms (*16–19*). Particle mass, composition, and size have all been cited as modulators of these responses. However, these animal studies have been performed using ambient particle surrogates, including fly-ash, acid sulfate aerosols, silica particles, iron oxide, or carbon, which may not be representative of ambient particle exposures.

Recent technological advances have made it possible to directly expose animals to concentrated ambient particles (CAPs). These methods include the Harvard ambient particle concentrator (HAPC) and the New York University concentrator (*20*). Studies using the HAPC have shown that CAPs exposures are associated with significant alterations in breathing patterns and acute inflammatory responses marked by neutrophil influx and increased vascular permeability in normal and chronic bronchitic rats (*22,23*). Gordon et al. (*24*) failed to find pulmonary responses but reported hematologic changes in rats, including elevated levels of polymorphonuclear leukocytes, after exposure to New York City CAPs. These initial studies illustrated detrimental effects of CAPs and indicated the utility and complexity of using CAPs in studies of particle effects.

In this paper, we present the results of novel studies in dogs assessing pulmonary inflammatory and hematologic responses to repeated exposures to CAPs over a large number of sample days with a comprehensive physicochemical characterization of the particles. The use of canines in these studies provides a genetically diverse large animal model as compared to previous studies carried out in rats. Performing repeated measures also provided an advantage over previous studies involving rodent responses to CAPs. The considerable day-to-day exposure variability in CAPs concentration and composition in conjunction with the relatively large number of experimental days made it possible to define responses to specific particulate components. In these studies, data are analyzed on several different levels including simple comparisons of treatment groups, linear regression analyses of day-by-day CAPs parameters versus responses, and factor analyses of CAPs elemental data in relationship to biologic outcomes to identify potentially harmful components of ambient particulate matter.

Materials and Methods

Animals. Dogs were purchased from Butler Farms (USDA 21-A-003; Clyde, NY), a U.S. Department of Agriculture-approved breeding facility for dogs for research. These mixed-breed dogs were intact female retired breeders less than 5 years old weighing between 14 and 17 kg. Upon arrival to our laboratory, a comprehensive veterinary examination was performed including physical examination and assessment of pulmonary health plus a clinical laboratory examination including a chemistry profile and hematologic and microbiologic assessments. The measured parameters were required to be within normal ranges for an animal to be included in the study (*25*). Dogs were housed and handled in accordance with National Institutes of Health and Harvard Animal Care Committee guidelines.

Preparation of canines for experimental procedures. All experiments were performed using pairs of dogs that were also housed together in a single kennel run. Dogs were

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paired after arrival at the Harvard School of Public Health facility. Dogs were acclimated to all procedures for several weeks before experiments.

Tracheostomy. We exposed dogs to CAPs or filtered air via permanent tracheostomies. Chronic tracheostomies were surgically created in each dog by the method of Dalgare et al. (*26*), as further refined by Nelson (*27*). Drazen et al. (*28*) have reported that tracheostomy per se does not affect airway mechanics. For tracheostomy surgery, dogs were fasted a minimum of 10 hr before anesthetic induction. Premedication/induction anesthesia used an intramuscular mixture of atropine (0.04 mg/kg), ketamine (10 mg/kg), and xylazine (1.5 mg/kg). Anesthesia was maintained with halothane gas administered via a semi-closed rebreathing system (using an OHIO Heidbrink Kinet-o-meter anesthesia machine; Ohio Medical Products, Madison, WI) via an endotracheal tube.

The tracheostomy surgical site was centered on the ventral midline of the neck and extended from 1–2 cm cranial to the larynx to a point just past the thoracic inlet, and 10–15 cm laterally on either side of the midline. The medial edges of the sternohyoid muscles were sutured to the fascia of the dorsolateral-lateral aspect of the trachea to decrease the tension on the skin to mucosa anastomosis and thereby decrease the tendency for dehiscence. An H-shaped incision in the full thickness of the tracheal wall was made centrally between the fourth and fifth tracheal rings. The two tracheal wall flaps were then reflected outward while the skin flaps were reflected inward, and the skin was sutured to the mucosa around the entire tracheostomy. Following the procedure, a jacket with a high neck was placed on the dog to protect the tracheostomy site. The tracheostomy healed completely within 2 weeks with a permanent stoma, which required minimal maintenance. For exposures, a modified, cuffed tracheostomy tube was inserted into the stoma.

Canine chamber training. After tracheostomies healed, we acclimated the dogs to the exposure chamber over a 2–4-week period before the first exposure to minimize stress associated with an exposure. Evaluation of dog comfort with each training step was subjectively determined by a veterinarian. Training began with simply bringing the dogs to the inhalation laboratory and allowing them to interact with each other and with their caretakers. Next, the dogs were placed in the chamber without concentrator pumps operating for 1–2 hr, gradually prolonging the chamber time up to 6 hr. The 1,000-L chamber was made of stainless steel with a Plexiglass door (*29*). The animals were separated from each other by a metal wire enclosure that allowed them free range of movement to stand, sit, or move from side to side. In the next training step, the tracheostomy tube (6 or 7 mm inner diameter depending on the size of animal and of tracheostomy stoma) was inserted for 1–2 hr. In the next stage, the cuff of the tracheostomy tube was inflated and left in place for 4–6 hr. The next advance in training was to turn on the HAPC airflow and attach the inspiratory and expiratory breathing tubes to the tracheostomy tubes. Then, the breathing tubes were fastened around the neck of the dog and taped together in the back such that the two tubes encircle the neck. We kept the animals in the chamber initially for 2–3 hr, and increased the time to a full 6 hr. All steps were repeated until the dogs appeared relaxed in this setting before exposures were undertaken.

Harvard ambient fine particle concentrator function. We used the Harvard ambient fine particle concentrator (HAPC) to expose animals to concentrated ambient fine particles (0.1–2.5 μm). This system has been described in detail elsewhere (*20*,*30*). Briefly, the HAPC used for the animal inhalation studies consists of three components: a high-volume, 2.5-μm selective inlet, a series of three virtual impactors with a 0.1-μm size cutoff (concentrator stages I, II, and III), and the animal exposure chamber.

The inlet is a conventional high-volume impactor (Fractionating Sampler; Anderson, Inc., Atlanta, GA) with an upper size cut of 2.5 μm at 5,000 L/min. The deflected flow of the conventional impactor was drawn through a series of three virtual impactors. Particles 0.1–2.5 μm were concentrated by the virtual impactors; the total concentration factor for the three virtual impactor stages was between 17 and 28. The minor flow of the third stage containing the CAPs was 50 L/min and was split into two fractions, 40 and 10 L/min. The first (40 L/min) was supplied to the animal exposure chamber and the second (10 L/min) was used for chemical and physical characterization of the particles.

The concentrated aerosol from the stage III of the virtual impactor was supplied to each dog receiving a CAPs exposure via breathing tubes. Sham exposures were also performed in the exposure chamber using the same attached breathing tubes at the same pressure and flow rate. For sham exposure, ambient air was filtered using a glass fiber filter (collection efficiency: 99.6–99.9%; Type A/E; Gelman, Ann Arbor, MI). The use of the three virtual impactor stages results in a pressure drop of about 10 inches of H_2O. Because the air pumping units were downstream from the concentrator/inhalation chamber, both the chamber and the breathing tubes were operated under a matched negative pressure (~ 10 inches H_2O). The residence time of the aerosol inside the virtual impactors was several seconds. The aerosol residence time in the breathing tubes was about 10 sec, resulting in minimal particle losses on the tubes, which was confirmed by upstream and downstream measurements.

Exposure characterization. We conducted animal exposures to CAPs in the inhalation facility at the Harvard School of Public Health. Outdoor air was drawn through a manifold into the HAPC. Exposures typically took place between 0900 and 1500 hr each day. For continuous measurements of concentrations of CAPs mass, we used a tapered element oscillating microbalance (TEOM) (*31*) and for black mass we used an aethalometer (model AE-14; Magee Scientific Inc., Berkeley CA) (*32*). These measurements were integrated over 5-min intervals throughout the 6-hr exposure period. We determined all other CAPs physical and chemical characterization measurements by filter-based samples collected over the entire 6-hr exposure period as described below.

The ambient and chamber levels of particulate mass were determined gravimetrically. Particles were collected on preweighed, 47-mm Savillex teflon filters (collection flow rate = 3 L/min). Filters were weighed using a Cahn 31 electrobalance in a temperature- and humidity-controlled room. We used the end filter weight, sampling time, and sampling flow rate to calculate the particle concentration in micrograms per cubic meter.

Concentrated particles were also collected on Teflon filters in parallel with a filter pack for measurement of sulfate, elemental and organic carbon, and elemental analysis. We determined sulfate concentrations using ion chromatography (*33*). Elemental analyses were conducted by X-ray fluorescence (XRF) (*34*) by Chester LabNet (Tigard, OR).

To determine the size distribution of ambient particles, samples were collected isokinetically from the concentrator inlet using a micro-orifice uniform deposit impactor (MOUDI; MSP Corporation, Minneapolis, MN). The MOUDI is a cascade impactor with micro-orifice nozzles, which collects particles onto preweighed filters at a flow rate of 30 L/min. We used the weights (after particle sampling) at each stage to determine the mass median particle diameter (MMD) and its geometric standard deviation (GSD). Weather data were obtained from the National Weather Service station at Logan International Airport (Boston, MA).

Experimental design. Inflammatory and hematologic responses after CAPS exposure were assessed using paired and crossover inhalation protocols, respectively. In paired experiments, two dogs were simultaneously exposed to either CAPs or filtered air (sham

exposure) for 6 hr on 3 consecutive days. Twenty-four hr after the third day of exposure, subjects underwent bronchoalveolar lavage to assess pulmonary cellular and biochemical responses. Thus, comparisons could be made between the same dogs exposed to CAPs and baseline or sham exposures. We used a total of eight different dogs in these experiments. In crossover experiments, one dog of a pair received CAPs exposure, while its partner was concurrently exposed to filtered air. The following week, the control dog of the pair was exposed to CAPs, and the other dog received filtered air. Therefore, for comparisons, each CAPs-exposed dog had a contemporaneous sham-exposed control as chambermate as well as herself as a sham-exposed control. All CAPs and filtered air exposures were conducted for 6 hr on 3 consecutive days. Before exposure and after each day of exposure, blood samples were drawn from individual animals. A total of 10 dogs were used in crossover analyses. Subsequent data analyses of these biologic responses examined relationships between biologic responses and CAPs parameters such as mass, size, and composition.

We used the same population of dogs in both paired and crossover protocols. Dog baselines were typically performed at least 1 month after the dogs arrived at the facility before inclusion in any experimental studies. After inclusion in any experimental design, dogs were allowed to recover for at least 3 weeks and typically several months between experimental protocols. The scheduling of the dog studies resulted in a random selection of exposure days that included exposures in all seasons; only heavy rain necessitated postponement or delay of an exposure. In both paired and crossover studies, we randomized the initial exposures (CAPs or filtered air) for individual pairs of dogs. A total of 20 different CAPs exposure days and 15 different sham exposure days were undertaken in the paired studies and a total of 24 exposure days were undertaken in the crossover studies.

Bronchoalveolar lavage and cellular analyses. We performed bronchoalveolar lavage (BAL) only in the paired studies because of the need for recovery time after the procedure. The procedure was carried out with the canines fully anesthetized as described for the tracheostomy surgery. In addition, the dogs were given intramuscular butorphanol (0.2 mg/kg) before lavage to suppress the cough reflex. The bronchoscope (Olympus BF Type 1T with Olympus CLE-3 light source; Olympus Optical Products, Tokyo, Japan) was inserted via an endotracheal tube for baseline studies and via the tracheostomy subsequently. In all studies, it was passed into the right mainstem bronchus, and then advanced into the right lung until it became wedged in a small airway. We used 50 mL of Dulbecco's 1X phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} for each lavage. The fluid of the lavages was retrieved via vacuum; the fluids from all lavages were pooled and kept on ice until processing at the end of the procedure. We repeated lavages until a minimum of 200 mL of lavage fluid was retrieved.

Lavage fluid was separated for analyses of total cell counts, differential cell profiles, and lavage supernatant protein levels. We used a 25 μL aliquot of the BAL to generate a total cell count and cell viability. Duplicate, well-mixed samples (100 μL) of the BAL return were cytocentrifuged onto microscope slides (Cytospin 2; Shandon Southern Instruments, Sewickley, PA), air dried, and stained with Wright Giemsa stain (VWR Stat Stain, Brisbane, CA). From these slides, we performed a differential count of 300 cells using standard morphologic criteria (*35*). The remaining BAL fluid was centrifuged (800*g* for 10 min), and a 1-mL aliquot of the supernatant was stored at –70°C for subsequent protein analysis; the remaining supernatant was discarded. We measured total protein levels in BAL fluid via the method of Bradford (*36*) using commercially available protein detection reagents (Pierce, Inc., Rockford, IL).

Blood sampling. Before a 3-day exposure and immediately after each day of exposure, a 1-mL and two 3–4 mL venous blood sample was taken from each dog. The smaller sample was collected into a Vacutainer tube with sodium citrate (Becton-Dickinson, Franklin Lakes, NJ) and sent to Tufts Veterinary Diagnostic Laboratory (Grafton, MA) for cell counts and differential analyses. The larger sample was taken for fibrinogen analysis. This blood sample was also collected into a tube containing sodium citrate. These samples were immediately transported to Geoffrey Tofler's laboratory at Harvard Medical School, where plasma fibrinogen levels were determined using the von Clauss method, a functional assay for thrombin-clottable fibrinogen. Briefly, dilute plasma was mixed with a thrombin solution of a constantly high concentration, and the clotting time of the mixture was measured. Fibrinogen concentration, which is inversely proportional to the clotting time, was determined from a standard curve. An ST4 Coagulation Instrument (Diagnostic Stage; Diagnostica Stago, Asniéries, France) was used to measure clotting time.

Data analyses. We applied multiple levels of analysis to our data. It is important to note that BAL data were from paired experiments only, and hematologic data were from crossover experiments. In preliminary BAL analyses, differences between exposure groups (baseline, sham, CAPs) were compared using repeated-measures analysis of variance (ANOVA) tests. A *post-hoc* Student-Neumann-Keuls analysis was used to compare groups.

Exploratory graphical data analyses of BAL and hematologic data from paired and crossover studies were conducted. Biologic responses were plotted versus daily CAPs exposure parameters (i.e., mass, sulfate, carbon, and elemental concentrations). For crossover studies, simultaneously obtained blood parameters from sham-exposed animals were plotted as daily exposure controls. Fitted values from linear regression analyses were plotted to identify potential associations between elemental concentrations and response data.

We applied mixed linear regression models to both BAL and hematologic responses to estimate differences due to exposure while controlling for random animal effects. All models were fit using PROC MIXED of SAS (SAS Institute, Cary, NC). Biologic response data representing proportions were arcsine transformed before model fitting to normalize the distribution and stabilize variance (*37*). For BAL analyses, for which data consisted entirely of paired exposures, differences between baseline, sham, and CAPs responses were related to exposure while controlling for random animal effects. For blood data, from crossover exposures, models were fit relating differences between a response to exposure, controlling for an animal's baseline as well as random animal and day effects. Both CAPs concentration and composition characterized exposure dose in the above models. Because only one BAL measurement was taken following each 3-day exposure, we considered both the 3-day mean and final day concentrations in the BAL models. Day-specific postexposure blood values were related to exposure concentration for the same day.

To characterize elemental composition in both the BAL (paired) and blood (crossover) data sets, we applied principal components analyses (PCA) to particle elemental concentration data for both the paired and crossover studies. The factor scores and loadings were calculated by diagonalizing the correlation matrix, and the extracted factors were rotated using the Equamax rotation procedures (orthogonal factor rotation; SPSS 8.0 Software; SPSS, Inc., Chicago, IL). Factor rotations were also conducted using oblique rotations (*38*). Orthogonal and oblique rotations yielded similar results, and we used the orthogonal rotations in the further analysis. Finally, we determined the absolute factor scores using the method described by Koutrakis and Spengler (*38*). In subsequent regression

analysis of daily factor scores versus biologic responses, regression coefficients were considered significant for associated p-values < 0.05.

Results

Repeated-Measures ANOVA by Treatment Group

Mean percentages of BAL macrophages (MAC), polymorphonuclear leukocytes (PMN), lymphocytes (LYM), eosinophils (EOS), and total BAL supernatant protein are shown in Table 1. There were no significant differences between baseline, sham, or CAPs groups among all percentages (Table 1). Although the percentage of PMN doubled in the CAPs-exposed group, the difference was not statistically significant ($p = 0.07$). Comparison of the baseline, sham, and CAPS protein did not indicate significant alteration. However, mean total BAL protein was significantly higher after the CAPs exposures compared to sham ($p < 0.0001$).

Mean white blood cell parameters [including total white blood cell count (WBC), percent blood polymorphonuclear leukocytes (PMN), percent blood lymphocytes (LYM), percent blood monocytes (MONO), percent blood EOS, and plasma fibrinogen levels] were not altered by CAPs exposure (Table 2).

Mean red blood cell parameters [including total red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), and platelet counts (PLT)] were not altered by CAPs exposure (Table 3). Fibrinogen levels were significantly affected by the day of exposure, but not the type of exposure itself ($p < 0.05$; Table 4).

Exposure Characterization

Daily ambient and CAPs measurements are listed in Table 5. For all of the experimental days reported here, the particle size had little variability and ranged from 0.2 to 0.3 µm mass median aerodynamic diameter. In contrast, the exposure data in Table 5 show considerable day-to-day variability in both mass and composition of CAPs. Individual elemental measurements in CAPs samples from paired and crossover exposures are shown in Table 6.

Exposure Response versus Particle Characterization

Exploratory graphical analyses. We performed exploratory graphical linear regression analyses to identify associations between biologic responses and CAPs mass, component data, or weather parameters from paired and crossover experiments. CAPs exposure parameters examined in these studies included integrated CAPs mass, CAPs sulfate mass, fine CAPs mass, black carbon, CAPs elemental mass concentrations (Al, Si, S, Ca, Ti, V, Cr, Mn, Fe, Ni, Cu, Zn, Br, Pb, Ba), ambient temperature, relative humidity, and barometric pressure.

Several CAPs component parameters appeared to be qualitatively associated with altered biologic responses. For example, increased Al concentration was associated with increased percentages of blood PMN and decreased LYM in the CAPs-exposed dogs (Figure 1A). Increased V was associated with increased percentages of blood PMN in the CAPs-exposed dogs (Figure 1B). In both cases, the sham-exposed chamber-mates did not show changes regarding date-matched AL (Figure 1A) or V (Figure 1B) exposure levels. The scatter of the data points indicates the day-to-day variability associated with responses. Weather data were not associated with alterations in biologic responses

Table 1. BAL parameters obtained in paired experiments related to animal exposure.

Treatment group	% MAC	% PMN	% LYM	% EOS	Total protein (µg/mL)
Baseline	85.1	9.1	4.8	1.1	307.7
(n = 20–26)[a]	(1.9)	(1.9)	(1.0)	(0.5)	(42.3)
Sham exposure	88.6	7.6	3.2	0.6	164.2
(n = 3–5)	(1.9)	(1.5)	(1.3)	(0.3)	(17.9)
CAPs exposure	81.7	14.5	2.8	1.0	362.0*
(n = 10–12)[a]	(4.7)	(4.7)	(0.5)	(0.2)	(46.8)

The data represent the mean (SE) for each exposure (n > 5 per group).
[a]The smaller values for the sample numbers were all related to the number of total protein measurements; all cell differential percentages were determined using the maximum sample number. *p < 0.05 in the comparison of CAPs and filtered air responses.

Table 2. White blood cell differential numbers and percentages on each day of CAPs or sham exposures.

Treatment	PMN		LYM		MONO		EOS	
day	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs
Number								
Baseline	6,262	6,148	1,655	1,778	295	373	603	447
(n = 12–15)	(479.2)	(513.0)	(104.3)	(111.8)	(47.6)	(78.5)	(87.5)	(63.4)
Day 1	7,344	7,081	1,699	1,739	419	382	637	446
(n = 10–11)	(477.3)	(686.0)	(104.1)	(71.85)	(68.8)	(61.7)	(102.4)	(74.3)
Day 2	5,805	6,593	1,873	2,150	381.7	345	601	528
(n = 10–12)	(507.6)	(448.1)	(117.3)	(144.4)	(54.5)	(53.3)	(66.8)	(83.7)
Day 3	6,486	5,945	1,891	2,043	357.4	383	553	430
(n = 11–13)	(434.5)	(397.2)	(141.1)	(166.4)	(48.8)	(32.4)	(82.7)	(67.22)
Percentages								
Baseline	69.8	68.7	19.4	21.5	3.3	3.6	7.3	6.2
(n = 15–16)	(2.1)	(1.6)	(1.4)	(1.4)	(0.5)	(0.6)	(1.3)	(0.9)
Day 1	73.8	70.7	16.6	20.3	3.9	3.4	5.8	5.7
(n = 12–13)	(1.8)	(2.1)	(1.2)	(1.7)	(0.6)	(0.4)	(1.0)	(0.9)
Day 2	68.6	67.8	20.6	22.4	4.1	3.4	6.7	6.4
(n = 12–14)	(2.2)	(1.5)	(1.4)	(1.4)	(0.5)	(0.8)	(0.8)	(1.0)
Day 3	69.0	69.7	20.8	21.0	3.7	3.7	6.3	5.5
(n = 15)	(2.3)	(1.9)	(1.6)	(1.6)	(0.6)	(0.4)	(1.0)	(0.7)

The data represent daily mean (SE) for each exposure. There are no significant exposure or day-to-day differences. Cell count data were presented as the total daily count and daily percentage for each cell type (n > 10 per group).

Table 3. Red blood cell parameters [daily mean (SE) of 6 samples] related to animal exposures obtained in crossover experiments related to animal exposure.

Treatment	RBC (10^6/mL)		HGB (g/dL)		HCT (%)		MCV (fL)		PLT (10^3/µL)	
day	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs	Sham	CAPs
Baseline	6.74	7.11	15.05	15.9	42.5	44.83	65.32	64.77	279.00	284.50
	(0.29)	(0.22)	(0.50)	(0.47)	(1.54)	(1.58)	(1.29)	(1.24)	(20.81)	(16.80)
Day 1	6.84	6.97	15.47	15.58	44.67	45.17	68.00	67.37	304.00	287.50
	(0.15)	(0.20)	(0.24)	(0.34)	(0.67)	(0.98)	(0.92)	(0.56)	(17.13)	(15.17)
Day 2	6.72	7.06	15.18	15.80	43.50	46.17	67.65	67.52	297.83	272.33
	(0.15)	(0.12)	(0.36)	(0.21)	(0.76)	(0.48)	(0.96)	(0.55)	(20.40)	(14.23)
Day 3	6.66	6.80	15.02	15.30	43.83	43.83	68.23	68.18	285.83	259.33
	(0.12)	(0.16)	(0.22)	(166.4)	(0.60)	(1.08)	(1.15)	(0.89)	(13.80)	(19.72)

Animals were assessed before treatment, after each day of sham exposure, and after each day of CAPs exposure. There were no significant exposure or day-to-day differences in either CAPs and sham samples.

in exploratory graphical analyses of either paired or crossover experiments.

Mixed linear regression analyses. Based on these exploratory graphical analyses, we performed regression analyses controlling for dog-to-dog variability to identify associations between biologic responses and individual CAPs mass and elemental data. Exposure data from paired experiments were analyzed to evaluate CAPs parameters for 3-day average and third-day-only exposures. The data of these analyses are not shown, but the results of these analyses are presented descriptively. Significance was set at $p < 0.05$. Three-day average concentrations of two elements from paired exposures, Al and Ti, were significantly associated with dose-dependent decreases in BAL MAC and increases in PMN differential percentages. None of the third-day-only individual elemental concentrations were associated with any changes in BAL parameters.

In analysis of crossover study hematologic data evaluating daily CAPs parameters, a number of significant CAPs-dependent alterations were observed and are reported qualitatively in Table 7. CAPs sulfate concentrations were associated with an increase in WBC counts; black carbon (BC) concentrations were related to increased PMN and decreased EOS; CAPs mass measurements were associated with decreases in blood EOS; Ti and Zn were associated with increased PMN; Al, Mn, and Si were linked to increased PMN and decreased blood LYM; V, Fe, and Ni were related to increased PMN; and Na was associated with increased blood LYM. Significantly decreased PLT counts were associated with total CAPs mass. There were no significant alterations in RBC parameters, including total cell count, hemoglobin, hematocrit, and PLT counts, related to any of the elemental concentrations. Thus, these analyses using additional control of dog-to-dog variability identified a number of components to which biologic responses may be significantly related. However, this analysis cannot differentiate among these individual components which may be correlated themselves. Therefore, we perfomed factor analysis of the CAPs elemental data in an effort to investigate relationships between biologic effects and groups of related elements.

Biologic responses versus factor scores. Four factors were identified from the paired exposure experiments (Table 8) rotated using equimax procedures: a V/Ni factor, an S factor, an Al/Si factor, and a Br/Pb factor.

Factor analysis was also applied to the crossover exposure elemental data, and six factors were identified (Table 9): a V/Ni factor, an S factor, an Al/Si factor, a Br factor, a Na/Cl factor, and a Cr factor. Black carbon, which was measured only in the crossover exposure studies, shared some association with the V/Ni, S, and Br factors.

We determined factor scores for the paired and crossover experiments. The concentration of individual elements associated with the identified factors were calculated for each exposure (Tables 10 and 11). As can be observed, the estimated and measured concentrations were generally in good agreement and are comparable to similar analyses of larger data sets for air particulate in the eastern United States (*38*).

The biologic responses were regressed on daily factor scores controlling for dog-to-dog variation. Additionally, in crossover studies, the data were controlled for day-to-day variation in biologic responses above that explained by CAPs exposure. We compared changes in cell percentages obtained from BAL in paired exposures to 3-day CAPs exposure averages (Figure 2A) and separately to the third (most proximal) day of exposure only (Figure 2B). The Al/Si factor was associated with an increased percentage of BAL PMN with reductions in the percentage of BAL MAC, LYM, and EOS. Although the S factor was associated with a decreased percentage BAL PMN and an increased

Table 4. Total concentration of fibrinogen.

Treatment day	Fibrinogen (µg/mL) Sham (n = 3–4)	CAPs (n = 8)
Baseline	228.8 (12.4)	231.6 (26.6)
Day 1	233.8 (23.2)	259.3 (21.3)
Day 2	299.5* (41.8)	285.5* (16.1)
Day 3	318.0* (65.1)	311.9* (26.1)

Fibrinogen is expressed as a total concentration for each day (n > 3 per group). There were significant day-to-day differences in fibrinogen levels in both CAPs and sham samples.
*p < 0.05 versus exposure day.

Table 5. Measured exposure parameters for all studies.

Exposure parameter	Exposure type	Mean ± SD
MMAD (µm)	Paired	0.24 ± 2.7[a]
	Crossover	0.27 ± 2.3[a]
Ambient mass (µm)	Paired	12.31 ± 8.48
	Crossover	12.36 ± 7.77
Ambient sulfate (µg/m^3)	Paired	3.43 ± 2.93
	Crossover	2.83 ± 1.96
CAPs mass (µg/m^3)	Paired	203.40 ± 147.30
	Crossover	360.80 ± 266.60
CAPs sulfate (µg/m^3)	Paired	53.08 ± 43.15
	Crossover	85.18 ± 70.58
Mass conc factor	Paired	17.27 ± 1.45
	Crossover	28.29 ± 1.52
Sulfate conc factor	Paired	16.42 ± 1.45
	Crossover	28.14 ± 1.80
Black carbon mass (µg/m^3)	Paired	NA
	Crossover	11.92 ± 8.46
TEOM $PM_{2.5}$ (µg/m^3)	Paired	NA
	Crossover	299.0 ± 226.00

Abbreviations: conc, concentration; MMAD, mass median aerodynamic diameter; NA, not available. Paired values were from experiments involving simultaneous CAPs exposure of two dogs. Crossover values were obtained during experiments where one dog received CAPs, while a partner was simultaneously exposed to filtered air. Ambient values represent ambient atmospheric concentrations; CAPs values represent concentrations achieved using the HAPC. The mass and sulfate concentration factors illustrate the concentration achieved over ambient levels using the HAPC. Black carbon and TEOM $PM_{2.5}$ concentrations were continuous measures on concentrated particulate.
[a]σg value.

Table 6. Elemental concentrations in measured samples.

Element	LOD[a] (µg/m^3)	Precision[b] (%)	Paired average concentration (µg/m^3)[c]	Crossover average concentration (µg/m^3)[c]
Na	0.249	11.3	0.569 ± 0.987	2.313 ± 5.499
Al	0.049	18.6	0.680 ± 0.600	1.69 ± 1.500
Si	0.037	6.4	2.760 ± 1.910	5.740 ± 3.420
S	0.045	2.9	19.100 ± 18.610	28.380 ± 22.960
Cl	0.051	4.5	0.445 ± 0.106	4.811 ± 12.940
K	0.018	5.7	1.121 ± 0.211	2.015 ± 1.129
Ca	0.016	2.5	1.711 ± 1.599	2.723 ± 1.955
Ti	0.014	5.2	0.359 ± 0.844	0.307 ± 0.153
V	0.013	11.5	0.105 ± 0.132	0.147 ± 0.147
Cr	0.008	33.6	0.007 ± 0.010	0.0146 ± 0.011
Mn	0.012	8.2	0.0752 ± 0.056	0.1342 ± 0.058
Fe	0.010	4.3	2.934 ± 2.774	5.235 ± 2.739
Ni	0.006	11.6	0.070 ± 0.071	0.1183 ± 0.117
Cu	0.008	8.7	0.0953 ± 0.071	0.165 ± 0.068
Zn	0.006	4.6	0.335 ± 0.252	0.615 ± 0.392
As	0.010	51.4	0.010 ± 0.016	0.026 ± 0.023
Se	0.008	23.2	0.017 ± 0.023	0.029 ± 0.007
Br	0.008	5.7	0.054 ± 0.059	0.114 ± 0.106
Pb	0.021	10.9	0.123 ± 0.082	0.796 ± 0.314
Ba	0.286	25.8	0.604 ± 0.028	0.200 ± 0.104
Cd	0.058	–	0.019 ± 0.026	0.019 ± 0.028

Data are presented as mean ± SD.
[a]Limit of detection 2-σ interference-free detection limits supplied by Chester LabNet, based on 6-hr sampling at 3.0 L/min. [b]Percent precision based on replicate analyses. [c]Average ± SD of all samples analyzed by X-ray fluorescence; total of 20 paired exposure days and 24 crossover samples.

percentage BAL MAC, LYM, and EOS, these changes were not significant when 3-day averages were considered (Figure 2A). The V/Ni factor was associated with a relative increase in the percentage of BAL MAC and a decrease in the percentage of BAL LYM (Figure 2B). The Br/Pb factor was associated with a significant increase in neutrophils (Figure 2B). There were no significant changes in BAL total protein versus any of the factors.

Daily regression coefficients from biologic responses obtained in crossover experiments were compared to cellular and biochemical responses in blood separated into WBC and RBC parameters. WBC differential counts and WBC total counts related to atmospheric components showed several significant associations (Figure 3). The V/Ni and Al/Si factors were associated with significant increases in blood PMN and corresponding decreases in blood LYM (Figure 3A). The Na/Cl factor was associated with increased blood LYM. The Al/Si factor was also associated with a significant increase in total WBC counts (Figure 3B). In assessment of RBC parameters, the S factor was associated with significant decreases in both RBC counts and HGB concentration (Figure 4A). None of the factors was associated with significant differences in PLT numbers (Figure 4B).

Discussion and Conclusions

The present study investigated the effects of CAPs on pulmonary cellular parameters as well as hematologic parameters using a large-animal model. Normal, uncompromised dogs were investigated to ascertain effects of CAPs concentration and composition on these biologic parameters. This study used an ambient particle concentrator and a large number of exposure days in an attempt to link specific CAPs constituents with biologic responses.

Exposure data were quite variable in this study, and this variability facilitated analyses. The variability in particle constituent values (mass and elemental) is the result of source variations, weather, air mass trajectories, wind direction, and season. Therefore, the variability in both exposure data and biologic response had the potential to better define health effects. Initial comparisons of mean biologic responses did not show significant differences between treatment groups. However, there was substantial variability in response between exposures, suggesting it was important to relate response to day-specific exposure characteristics. We subsequently analyzed biologic responses to daily individual specific CAPs components by linear regression analyses to assess potential responses associated with exposure variability, as illustrated in Figure 2. These analyses did not control for dog-to-dog variability in the biologic response, which might lessen any

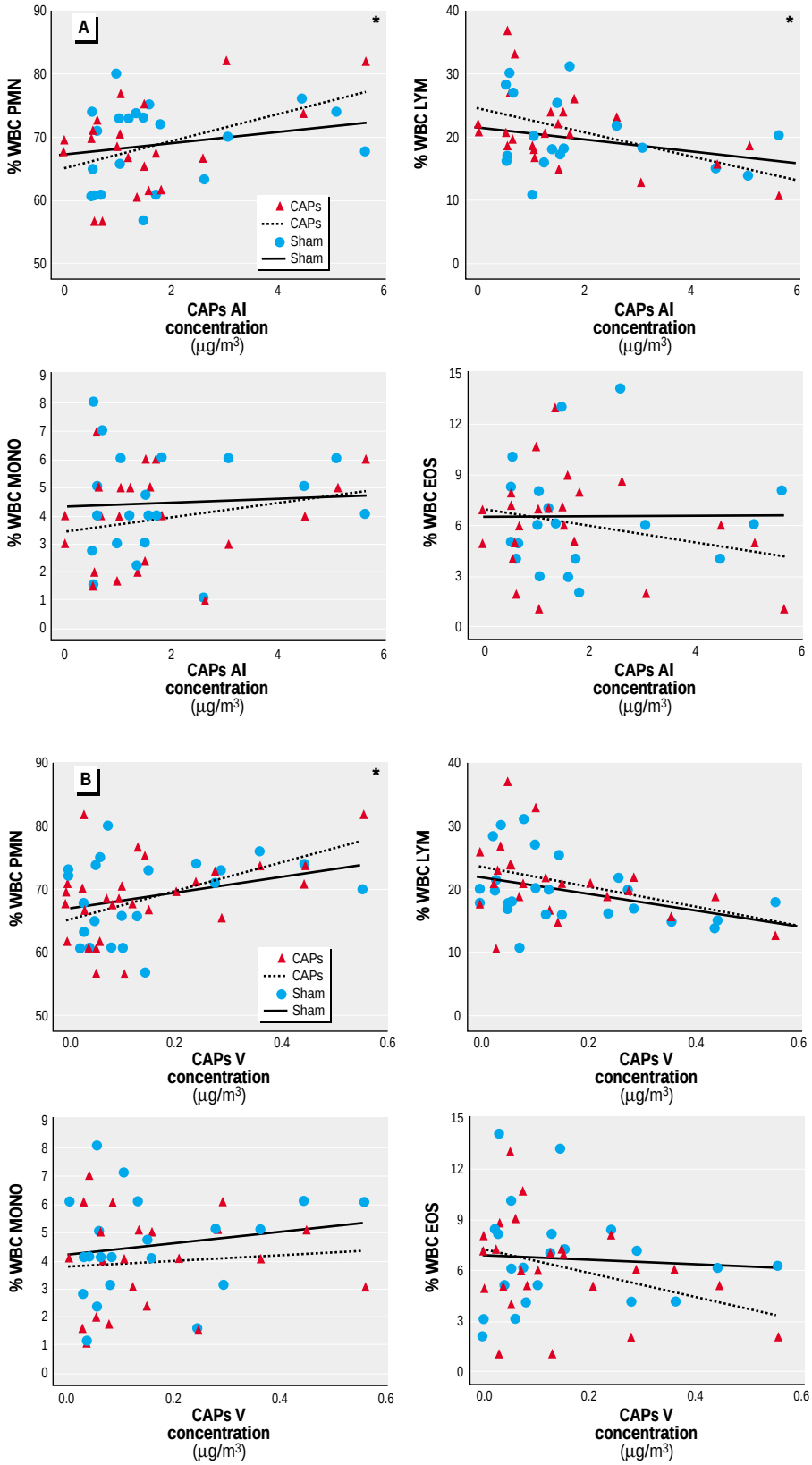

Figure 1. Exploratory graphical analyses illustrating changes in hematologic parameters related to CAPs elemental concentrations. Percentages of white blood cells from hematologic analysis after each day of CAPs or date-matched sham exposure analyzed by regression analysis of daily CAPs (*A*) Al concentrations and (*B*) V concentrations. Each data point represents a daily assessment.
*$p < 0.05$ versus CAPs regression.

apparent relationship. Mixed linear regression models controlling for dog-to-dog variability yielded profiles of responses to specific elements similar to the more simple models. This suggests that dog-to-dog variation plays a lesser role than day-to-day exposure variability in determining biologic responses. It was not possible to ascertain which individual CAPs components were most important in the biologic response because many of the components with similar response relationships are commonly associated in the atmosphere. Factor analyses of the elemental data were used to identify which CAPs elemental components are specifically associated in this data set and which of these factors (associated with specific elements) provide the basis for the biologic responses.

In previous experiments with CAPs, increased neutrophils in BAL from animals have been reported after 3-day exposure to CAPs (*22*). Alterations in hematologic profiles marked by increased circulating lymphocytes have been associated with CAPs exposure (*22*,*39*). These studies were performed in rats, which may be a species more sensitive to CAPs, but exposure days were limited ($n < 10$). CAPs exposure to rats has not consistently resulted in significant inflammatory responses as the number of exposure experiments have increased (*24*).

Interpretation of the pulmonary inflammatory responses observed in the present study is consistent with the rat data. Mean CAPs versus sham exposures exhibited no significant changes in BAL parameters. However, initial analyses of CAPs components showed that Al and Si were correlated with increased pulmonary neutrophil percentages. This association with the Al/Si factor components (associated with crustal sources) was maintained when data were controlled for dog-to-dog differences. This modest pulmonary inflammatory association suggests that pulmonary responses of normal subjects to increased ambient particulate matter are likely to be subtle.

The significant BAL responses related to the Al/Si factor raise a number of issues. It should be noted that the particle size measured in the present exposures was much smaller than might normally be found in crustal dust samples. However, it is possible that the elements of the Al/Si Factor are a surrogate of other particles that may be known respiratory irritants (*40*). In contrast to the present results, assessment of the pulmonary inflammatory effects of primary crustal elements usually shows no effect. Silicon dioxide can cause serious lung disease, but at much higher, occupational concentrations than found in the CAPs samples in this study (*41*). Aluminum is generally not considered an airborne hazard; likewise, titanium-rich particle concentrations far higher than those observed in the present study are considered necessary to induce biologic responses (*42*,*43*). Indeed, titanium dioxide is often used as a negative control in studies investigating particle inhalation effects. Elemental iron contributed primarily to the Al/Si and Br/Pb in the present study, not the V/Ni factor (see Table 6). Further studies of these constituents in this size range are needed.

The relationship of changes in BAL to 3-day average concentrations versus the last day of exposure concentrations produced different results. At this point, it is not clear which metric is more predictive of response. It is known that timing of pulmonary inflammatory responses is important. For example, some forms of the same materials produce early responses, while others have a more delayed response (*44*). It is possible that the particle constituents associated with

Table 7. CAPS mass and component predictors of hematologic responses assessed by mixed linear regression analyses.

Biologic response	Predictor
↑ WBC	Sulfate
↑ PMN	BC, Al, Mn, Si, Zn, Ti, V, Fe, Ni
↑ LYM	Na
↓ LYM	Al, Mn, Si
↓ EOS	CAPs mass, BC

BC, black carbon. These predictors had significant ($p < 0.05$) associations in the analyses.

Table 8. Rotating factor analysis loadings of elemental composition from CAPs samples obtained during paired exposures (20 days of measurement).

Element	V/Ni factor	S Factor factor	Al/Si factor	Br/Pb factor
V	0.967*	0.118	0.089	0.178
Ni	0.959*	0.085	0.115	0.216
S	0.079	0.912*	0.028	0.398
Al	0.077	–0.042	0.980*	–0.065
Si	0.105	0.173	0.954*	0.184
Ca	0.149	0.034	0.944*	0.146
Br	0.226	0.488	0.042	0.806*
Pb	0.240	0.351	0.110	0.874*

*Represents a significant association, which then defines the specific factor.

Table 9. Rotating factor analysis loadings of elemental composition from CAPs samples obtained during crossover exposures (24 days of measurement).

Element	V/Ni Factor	S Factor	Al/Si Factor	Br Factor	Ma/Cl Factor	Cr Factor
V	0.883*	0.151	0.257	0.299	0.016	0.176
Ni	0.928*	0.078	0.219	0.156	0.004	0.234
S	0.035	0.914*	–0.071	0.359	–0.113	0.101
Al	0.161	–0.128	0.959*	–0.080	–0.060	0.085
Si	0.227	0.107	0.915*	0.183	0.002	0.212
Br	0.165	0.415	0.012	0.880*	0.140	–0.006
Na	0.042	–0.124	–0.089	0.023	0.973*	–0.098
Cl	–0.046	–0.034	0.029	0.061	0.986*	–0.024
Cr	0.145	0.085	0.124	0.016	–0.063	0.975*
Black carbon	0.528[a]	0.534[a]	0.102	0.555[a]	–0.139	0.197

[a]Black carbon was weakly associated with the V/Ni, S, and Br factors. *Represents a significant association, which then defines the specific factor.

Table 10. Elemental and total mass concentrations (μg/m^3) from paired exposure days calculated from specific rotated factor analyses.

Element	V/Ni factor	S factor	Al/Si factor	Br/Pb factor	r^2	Calculated mass (μg/m^3)	Measured mass (μg/m^3)
Total mass	17.45	20.49	—	167.29	0.92	205.23	203.42
S	0.80	2.96	0.39	11.94	1.00	16.09	19.10
Si	—	—	2.91	—	0.92	2.91	4.14
Fe	—	—	1.13	2.53	0.89	3.65	2.93
Ti	—	—	0.50	—	1.00	1.69	2.00
Ca	0.11	0.02	0.92	0.43	0.94	1.48	1.71
Al	—	—	1.78	—	0.96	1.78	1.33
K	—	—	0.41	0.71	0.90	1.13	1.12
Ba	0.04	0.01	0.02	0.39	0.94	0.46	0.60
Zn	—	0.03	—	0.31	0.92	0.34	0.33
Pb	0.01	0.01	—	0.11	0.84	0.13	0.12
V	0.06	—	—	0.04	0.98	0.10	0.10
Cu	—	—	—	0.10	0.78	0.10	0.10
Mn	—	—	0.02	0.05	0.79	0.08	0.08
Br	—	—	—	0.08	0.91	0.08	0.08
Ni	0.03	—	—	0.03	0.99	0.06	0.07
Se	—	—	—	0.02	0.62	0.02	0.02
Cd	—	—	—	0.01	0.09	0.01	0.02
Cr	—	—	—	0.01	0.79	0.01	0.01
As	—	—	—	0.01	0.44	0.01	0.01

The r^2 value indicates the strength of the relationship for individual measured and calculated concentrations. Note the contribution of elements and the similarity between measured and calculated values.

the Al/Si factor may cause either a delayed or longer lasting, acute inflammatory response. The significant neutrophil increase associated with traffic sources in relationship to the third day's exposure data suggests that this increased response may be shortlived.

Our findings for peripheral blood provide evidence of extrapulmonary activity of inhaled particles. Previous work has suggested that ambient air particles exert significant effects on hematologic parameters after inhalation (*39*,*45*). In the present study, simple comparisons of exposure (CAPs, sham, or no treatment) were not associated with significant alterations in hematologic parameters. However, the alterations observed in peripheral blood parameters were related to specific particle constituents. Physiologically, it is unclear whether the extrapulmonary response is triggered by the response of the lung, or whether constituents of CAPs may become extrapulmonary to directly exert hematologic effects [reviewed by Godleski and Clarke (*46*)]. The increases in circulating neutrophils associated with constituents that may have differences in water solubility (V/Ni and Al/Si) suggest that distinction of mechanism of response may be important. The observed difference between the pulmonary and the hematologic responses to these two factors may have important implications in further understanding physicochemical properties of ambient particulate components in relationship to biologic responses.

Previous studies investigating relationships between constituents or surrogates of ambient air pollution and biologic responses have mainly focused on combustion-related trace metals (V, Ni, Cu) that are associated with airborne particulates (*14*,*15*). These studies have reported significant pulmonary inflammation caused by these elements alone or in mixtures. However, the use of instillation and *in vitro* techniques may deliver higher local doses compared to inhalation studies. Therefore, the difference in response to inhalation versus instillation exposure may simply be a manifestation of dose. In any case, the complex dosimetric features of real world ambient particles need to be carefully considered in the interpretation of all surrogate data.

The red blood cell changes associated with the S factor were also intriguing. Recent work has shown significant associations with changes in RBC and HGB in humans after exposure to elevated levels of ambient particles (*47*). The present work indicates the same result and suggests a relationship to a specific constituent of ambient particulate matter. The mechanistic basis and clinical impact of this observation are unclear, but these studies indicate that RBC parameters may also provide a sensitive indicator of biologic responses to ambient particles.

Overall, these studies provided new information on pulmonary inflammatory and hematologic responses to inhaled CAPs in a genetically diverse, large-animal model that could be assessed repeatedly. From these data, it appears that pulmonary inflammatory responses after ambient particle inhalation may not be a major contributor to the observed mortality and morbidity. In contrast, systemic changes marked by alterations

Table 11. Elemental and total mass concentrations (μg/m^3) from crossover exposure days calculated from specific rotated factor analyses.

Element	V/Ni factor	S factor	Al/Si factor	Br factor	Na/Cl factor	Cr factor	r^2	Calculated mass (μg/m^3)	Measured mass (μg/m^3)
Total mass	18.37	233.93	5.86	84.29	—	16.37	0.96	358.82	360.80
S	—	25.03	—	—	—	—	0.98	25.03	28.38
Black carbon	1.32	5.39	—	2.46	—	1.74	0.91	10.91	11.92
Si	—	—	3.90	—	—	—	0.84	3.90	6.40
Fe	0.35	0.27	2.18	0.31	—	1.54	0.94	4.64	5.24
Cl	—	—	—	—	7.32	—	0.97	7.32	4.81
Ca	—	—	1.57	0.24	—	0.66	0.65	2.46	2.72
Na	—	—	—	—	3.07	—	0.95	3.07	2.31
K	0.11	0.49	0.56	0.40	—	—	0.87	1.56	1.90
Al	—	—	1.82	—	—	—	0.92	1.82	1.69
Ba	—	—	—	—	—	0.14	0.19	0.14	0.81
Pb	0.02	0.03	0.05	—	—	0.02	0.73	0.13	0.80
Zn	0.08	0.13	0.13	0.07	—	0.15	0.86	0.56	0.60
Ti	0.02	0.03	0.13	0.01	—	0.01	0.92	0.21	0.31
Cu	0.01	0.02	0.02	0.01	—	0.03	0.95	0.09	0.16
V	0.04	0.03	—	0.02	—	0.03	0.92	0.11	0.15
Mn	0.01	—	0.03	—	—	0.03	0.73	0.07	0.13
Ni	0.03	—	0.03	0.01	—	0.03	0.96	0.10	0.12
Br	—	0.05	—	0.05	—	—	0.95	0.10	0.11
Se	—	0.02	—	0.01	—	—	0.33	0.02	0.03
Cr	0.00	—	—	—	—	0.01	0.98	0.01	0.02
As	0.00	0.00	0.00	0.01	—	—	0.97	0.02	0.02
Cd	—	0.01	0.00	—	—	—	0.05	0.01	0.02

Note the contribution of elements and the similarity between measured and calculated values. The r^2 value indicates the strength of the relationship for individual measured and calculated concentrations.

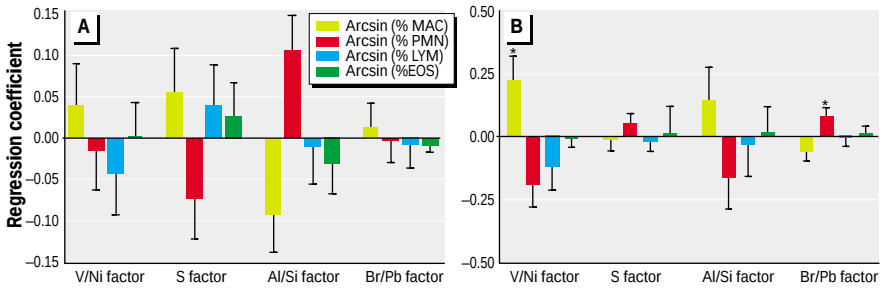

Figure 2. Regression values obtained by factor analysis of paired exposure data compared to (*A*) BAL parameters compared to 3-day exposure averages and (*B*) BAL parameters compared to third day-only exposure values. Data are controlled for individual dogs.
*$p < 0.05$.

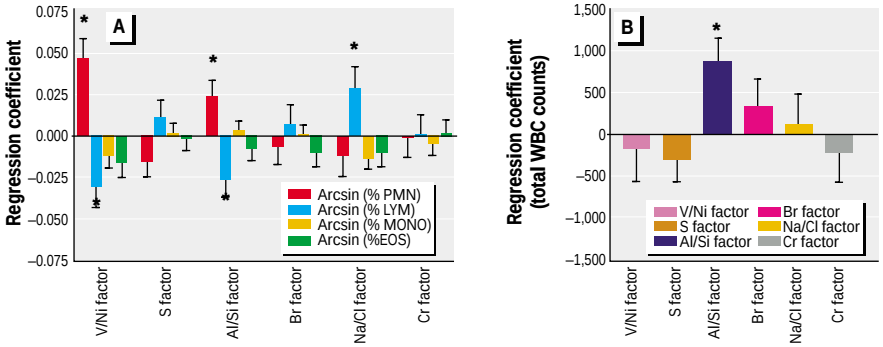

Figure 3. Regression values obtained by factor analysis of crossover exposure data compared to (*A*) percentages of white blood cells and (*B*) total WBC counts after each day of CAPs exposure. Data are controlled for individual dogs.
*$p < 0.05$.

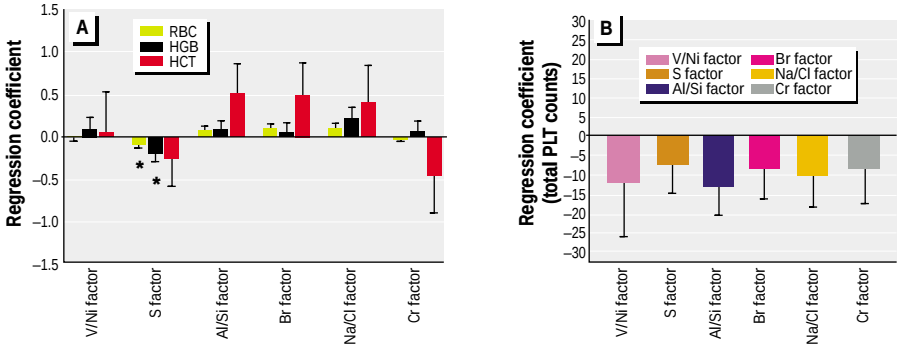

Figure 4. Regression values obtained by factor analysis of crossover exposure data compared to (*A*) blood parameters and (*B*) PLT counts after each day of CAPs exposure compared to sham exposures. Data are controlled for individual dogs.
*$p < 0.05$

in the hematologic profile may be a more sensitive indicator of particle-related biologic responses, which could be potentially linked to cardiac effects (*48*). An important finding of our study was the association of biologic responses with individual particle components rather than with fine mass. Although this was not surprising, it underlines the importance of developing a more specific exposure index. Finally, the design of these studies, using repeated measures on a population of dogs and a large number of exposure days, provided a useful approach to assess biologic responses to variant CAPs concentrations. Future work will focus on elucidating potential mechanisms for these biologic responses that may be related to epidemiologic studies of particle-associated mortality and morbidity.

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