

**Effects of Ozone Exposure on Cardiovascular Responses in Healthy and Susceptible Humans**

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## Table of Contents

	<u>Page</u>
List of Tables	6
Abstract	7
Executive Summary	8
Body of Report	10
References	26
Figure Legends	30
Tables	31
Figures	42
Glossary of Terms, Abbreviations, and Symbols	44

## List of Tables

	<u>Page</u>
Table 1. Subject characteristics: exercise study (n=25).	31
Table 2. Subject characteristics: ozone study (n=22).	32
Table 3. Exercise-induced changes in peripheral blood leukocytes, C-reactive protein, angiotensin-converting enzyme activity, and cytokines.	33
Table 4. Ozone-induced changes in peripheral blood leukocytes, C-reactive protein, and angiotensin-converting enzyme activity.	34
Table 5. Peripheral blood coagulatory changes with exercise.	35
Table 6. Peripheral blood coagulatory changes with ozone.	36
Table 7. Ozone-induced airway inflammation.	37
Table 8. Ozone-induced changes in lung function.	38
Table 9. Heart rate variability with exercise.	39
Table 10. Heart rate variability frequency-domain indices with exercise.	40
Table 11. Heart rate variability frequency-domain indices with ozone exposure.	41

## Abstract

Ozone (O<sub>3</sub>) is a major gaseous component of air pollution in urban environments. Recent epidemiological evidence suggests that O<sub>3</sub> exposure may increase cardiovascular morbidity, but the specific biological mechanisms mediating O<sub>3</sub>-associated cardiovascular effects remain to be elucidated. We designed a controlled human exposure study to address the question of whether short-term exposures to ambient levels of O<sub>3</sub> cause acute cardiovascular responses as assessed by heart rate variability (HRV) and systemic biomarkers of inflammation and coagulability. Twenty five subjects (15 healthy and 10 asthmatic subjects) were recruited to participate in the study. The subjects were exposed to three conditions (filtered air, 100 ppb O<sub>3</sub>, and 200 ppb O<sub>3</sub>) in random order. Exposures were for 4 hours with intermittent exercise (30 min of each hour) at a minute ventilation of 20 L/kg/m<sup>2</sup> body surface area. Exercise is used in controlled human exposure studies to simulate outdoor activity levels that involve higher ventilation rates that increase the effective dose of the pollutant. Heart rate variability was measured and blood samples were obtained immediately prior to, immediately after, and 20 h after each exposure period. Bronchoscopy to obtain bronchoalveolar lavage (BAL) fluid was performed at 20 h after exposure. We found that intermittent moderate-intensity exercise induced a pro-inflammatory systemic response characterized by increases in peripheral blood leukocyte counts, and C-reactive protein (CRP), monocyte chemotactic protein, and interleukin-6 concentrations. Exercise did not substantially alter levels of fibrinogen, angiotensin-converting enzyme, markers of coagulability, or HRV. We also found that exposure to 200 ppb O<sub>3</sub> induced a decrease in lung function, airway injury as manifested by an increase in total BAL protein, and airway inflammation as evidenced by increases in BAL neutrophils, eosinophils, and several pro-inflammatory cytokines (all as compared to control exposure to filtered air with intermittent exercise). Exposure to 100 ppb O<sub>3</sub> caused no decrease in lung function and only mild airway inflammation. Despite the induction of a local lung inflammatory response at 200 ppb, there was little evidence of a systemic inflammatory response to O<sub>3</sub>, with the exception of a trend toward an increase in serum CRP at 24 h after exposure. Moreover, no evidence of a pro-coagulatory response to O<sub>3</sub> exposure was found. Despite this lack of a systemic response, we did find that exposure to 200 ppb O<sub>3</sub> induced a significant decrease in the normalized high frequency (HF) domain of HRV at both 4 and 24 h after exposure. We also observed non-significant decreases in the HF domain with 100 ppb exposure at both the 4-h and 24-h time points. The presence of an exposure-relationship when the HF data at all concentrations and time points were analyzed in a linear regression also supports the role of O<sub>3</sub> exposure in the observed increase in HF HRV. To our knowledge this is the first controlled exposure study to find an effect on HRV with O<sub>3</sub> alone. The results suggest that short-term exposures to O<sub>3</sub> can have acute cardiovascular effects.

## Executive Summary

### Background

Ozone (O<sub>3</sub>) is a major gaseous component of air pollution in urban environments. While the acute pulmonary effects of O<sub>3</sub> inhalation have been extensively documented in previous studies, recent epidemiological evidence suggests that O<sub>3</sub> exposure may increase cardiovascular morbidity and mortality. Short-term increases in ambient O<sub>3</sub> concentrations have been associated with both an increase in hospital admission rates due to cardiopulmonary abnormalities as well as an increase in cardiovascular morbidity and mortality. The specific biological mechanisms mediating O<sub>3</sub>-associated cardiovascular effects remain uncertain.

One mechanism through which O<sub>3</sub> may affect cardiovascular health may be through alteration of cardiac sympathetic/parasympathetic autonomic balance. Heart rate variability (HRV), which describes the normal beat-to-beat variations mediated by cardiac autonomic function, is a biomarker of cardiovascular health. Decreased HRV, indicative of heightened sympathetic activity and decreased parasympathetic activity, is associated with decreased arrhythmia threshold and increased cardiovascular morbidity and mortality. Another potential mechanism of O<sub>3</sub>-related cardiovascular toxicity is the induction of systemic inflammation and a pro-coagulant state.

We designed a controlled human exposure study to address the question of whether short-term exposures to ambient levels of O<sub>3</sub> cause acute cardiovascular responses as assessed by HRV and systemic biomarkers of inflammation and coagulability.

### Methods

Twenty five subjects (15 healthy and 10 asthmatic subjects) were recruited to participate in the study. The subjects were exposed to three conditions (filtered air, 100 ppb O<sub>3</sub>, and 200 ppb O<sub>3</sub>) in random order. Exposures were for 4 hours with intermittent exercise (30 min of each hour) at a minute ventilation of 20 L/kg/m<sup>2</sup> body surface area. Exercise is used in controlled human exposure studies to simulate an outdoor activity pattern and increase the effective dose of the pollutant. Heart rate variability was measured and blood samples were obtained immediately prior to, immediately after, and 20 h after each exposure period. Bronchoscopy to obtain bronchoalveolar lavage (BAL) fluid was performed at 20 h after exposure. The data analysis was done in two steps. First, the effect of exercise on HRV variability and biomarkers of systemic inflammation and coagulability was evaluated by comparing data across the filtered air (FA) exposure. Second, the effect of O<sub>3</sub> on HRV parameters and the biomarkers of interest was investigated, using the data collected at the two different concentrations to assess whether there was evidence of exposure-dependent responses.

### Results

We found that intermittent moderate-intensity exercise induced a pro-inflammatory systemic response characterized by increases in peripheral blood leukocyte counts, and C-reactive protein (CRP), monocyte chemotactic protein, and interleukin-6 concentrations. While the total leukocytes increased at the 4-h time point and remained elevated at 24 h, there was no association between O<sub>3</sub> exposure and the change in leukocyte concentrations, suggesting that changes in leukocytes were mediated by exercise rather than O<sub>3</sub>. Exercise did not substantially alter levels of fibrinogen, angiotensin-converting enzyme, markers of coagulability, or HRV. We also found that exposure to 200 ppb O<sub>3</sub> induced a decrease in lung function, airway injury as manifested by an increase in total BAL protein, and airway inflammation as evidenced by increases in BAL neutrophils, eosinophils, and several pro-inflammatory cytokines (all as

compared to control exposure to filtered air with intermittent exercise). Exposure to 100 ppb O<sub>3</sub> caused no decrease in lung function and only mild airway inflammation.

Despite the induction of a local lung inflammatory response at 200 ppb, there was little evidence of a systemic inflammatory response to O<sub>3</sub>, with the exception of a trend toward an increase in serum CRP at 24 h after exposure. Moreover, no evidence of a pro-coagulatory response to O<sub>3</sub> exposure was found. Despite this lack of a systemic response, we did find that exposure to 200 ppb O<sub>3</sub> induced a significant decrease in the normalized high frequency (HF) domain of HRV at both 4 and 24 h after exposure. We also observed non-significant decreases in the HF domain with 100 ppb exposure at both the 4-h and 24-h time points. The presence of an exposure-relationship when the HF data at all concentrations and time points were analyzed in a linear regression also supports the role of O<sub>3</sub> exposure in the observed decrease in HF HRV.

## **Conclusions**

This controlled exposure study found a change in HRV with O<sub>3</sub> alone that is consistent with another recent study (Devlin et al. 2012). The strengths of this study include a relatively large number of subjects for a controlled human exposure study, careful assessment of potentially confounding exercise effects, use of two different concentrations of O<sub>3</sub> that allowed an exposure-response analysis, and measurement of biomarkers of both systemic and airway inflammation. Limitations include relative lack of power to study small changes (e.g., the trend toward an increase in CRP at 24 h after exposure might have become significant with a larger sample size) and study subjects that were relatively young and healthy. We chose to recruit such subjects for safety reasons, given that the effects of O<sub>3</sub> inhalation on cardiovascular endpoints in elderly subjects who might be at greater risk had not been previously studied. On the other hand, several previous studies of the effects of O<sub>3</sub> on pulmonary function in elderly subjects have shown them to be less sensitive than younger subjects. It is possible, however, that only patients with pre-existing atherosclerotic cardiac disease are at risk for O<sub>3</sub>-induced acute effects on cardiovascular outcomes other than HRV. We chose to study asthmatic subjects who were otherwise healthy as a potentially susceptible subgroup because of pre-existing airway inflammation. We and others have shown that asthmatic subjects have greater airway inflammatory responses to O<sub>3</sub>, but this may not translate to any increased risk for cardiovascular morbidity. Another caveat is that in order to deliver a sufficient effective dose of O<sub>3</sub> capable of causing mild airway inflammation, all exposures were with intermittent exercise.

Our results suggest that alterations in autonomic balance, which may reflect a nociceptive reflex response to airway irritation, may be one mechanism by which O<sub>3</sub> can cause cardiovascular morbidity. Our results also suggest that ambient levels of O<sub>3</sub> are unlikely to cause systemic inflammation and increased coagulability. A potential mechanism of O<sub>3</sub>-related cardiovascular toxicity that merits investigation is oxidative stress-induced endothelial dysfunction.

## Body of Report

### Introduction

Over the past two decades a large body of epidemiological evidence has linked exposure to ambient particulate matter (PM) to increased risk of mortality (Pope and Dockery 2006). Most of the excess mortality linked to PM is due to cardiovascular disease and a concerted effort was launched to fund research to elucidate the biological mechanisms by which inhaled particles lead to early cardiovascular death as well as intermediate endpoints on the pathway to early death. Less attention has been paid to acute cardiovascular responses to ozone ( $O_3$ ), at least in part due to the notion that  $O_3$  causes primarily local effects on the respiratory tract. However, with several recent epidemiological studies reporting increased risk of mortality associated with ambient exposure to  $O_3$ , new concerns about harmful cardiovascular effects of this pollutant have been appropriately raised.

Air pollution likely promotes cardiovascular events by multiple mechanisms. General pathways by which air pollution could cause vascular dysfunction have been reviewed by Brook (Brook 2008). Of these, a) the induction of systemic inflammation and/or oxidative stress and b) alterations in autonomic balance have the most plausible relationship to  $O_3$ . Initial responses to oxidant injury and inflammation could lead ultimately to endothelial dysfunction, acute arterial vasoconstriction, and pro-coagulant activity. Stimulation of nociceptive fibers in the airways may lead to changes in sympathetic and/or parasympathetic tone which could lead to arrhythmias.

Systemic inflammation in response to particle-induced local lung injury has been suggested as one potential mechanism of PM-related cardiovascular effects with oxidative stress as a likely cause of particle-induced lung injury (Brook 2008). An imbalance between the production of reactive oxygen species (ROS) and reactive nitrogen species and antioxidant capacity leads to a state of "oxidative stress" that contributes to the pathogenesis of multiple diseases (Dalle-Donne et al. 2006; Zhang 2010). The excess oxygen species can damage major cellular components, including membrane lipids, protein, carbohydrates, and DNA. Pathobiological consequences of this injury are inflammation and tissue damage. One mechanism by which oxidative stress promotes inflammation is through the activation of nuclear transcription factors, such as  $NF-\kappa\beta$ , which in turn induce the expression of multiple cytokine genes and initiate a cascade of inflammatory responses (Martinon 2010; Rizvi 2009).

Ozone is a prime example of an exogenous oxidizing agent that is capable of reacting with many biomolecules. While not a free radical,  $O_3$  has high redox potential. Most inhaled  $O_3$  reacts with components of the fluid layer that covers the respiratory epithelium, the respiratory-tract lining fluid (RTLF). The responses observed following exposure to  $O_3$  are thought to be mediated through oxidation reactions occurring within this air-fluid interface (Pryor 1994). The low solubility and high reactivity of  $O_3$  (Medinsky and Bond 2001) allow little of the gas to react directly with the underlying cells. Instead, its toxicity is transmitted to the respiratory epithelium by secondary oxidation products formed by the direct ozonization of RTLF lipids (Pryor et al. 1995). These lipid peroxidation species trigger the underlying cells to initiate inflammatory signals (Kafoury et al. 1999), resulting in airway neutrophilia that leads to secondary release of ROS with ensuing tissue injury. One controlled human exposure study documented increased airway oxidative stress after exposure to a 0.1 ppm  $O_3$  for 2 hrs (Corradi et al. 2002). If local airway oxidative stress can induce acute cardiovascular responses, inhalation of controlled concentrations of  $O_3$  should be a good model of this exposure-response in humans.

Autonomic imbalance is another potential mechanism of air pollution-induced cardiovascular dysfunction. Decreased parasympathetic nervous system activity and/or increased sympathetic nervous system activity could lead to acute vascular constriction and/or arrhythmias. Inhaled pollutants deposited in the respiratory tree can directly stimulate lung nerve reflexes via irritant receptors and, consequently, alter systemic autonomic balance. Heart rate variability (HRV) describes the normal beat-to-beat variations in heart rate that occur in response to changes in respiration and blood pressure, mediated by autonomic control through the baroreflex (deBoer et al. 1987; Kaushal and Taylor 2002). Multiple epidemiological studies have demonstrated an association between decreased HRV and increased risk of cardiovascular morbidity and mortality (Bigger et al. 1992; Dekker et al. 2000). Additionally, several epidemiological studies have shown an association between increased ambient particulate matter and/or ozone and decreased HRV (Liao et al. 2004; Lipsett et al. 2006; Pope et al. 1999). Heightened sympathetic activity and dampened parasympathetic activity are associated with decreased arrhythmia threshold and increased risk of malignant ventricular arrhythmias and death (Algra et al. 1993; Hohnloser et al. 1997). If the decreased HRV seen with inhalation of air pollutants is the result of sympathetic stimulation and parasympathetic attenuation, this may reflect a potentially adverse shift in cardiac autonomic balance. In other words, if short-term exposure to O<sub>3</sub> leads to decreased HRV, risk of cardiovascular morbidity would be increased.

Heart rate variability parameters are divided into two main types, time domain and frequency domain (Task Force 1996). Time domain parameters include SDNN, SDANN, SDNNI, and RMSSD. In a continuous electrocardiographic record, each QRS complex is detected, and the so-called normal-to-normal (NN) intervals (that is, all intervals between adjacent QRS complexes resulting from sinus node depolarizations) or the instantaneous heart rate is determined. The simplest variable to calculate is the standard deviation of the NN intervals (SDNN), that is, the square root of variance. Since variance is mathematically equal to total power of spectral analysis, SDNN reflects all the cyclic components responsible for variability in the period of recording. Other commonly used statistical variables calculated from segments of the total monitoring period are the following: 1) SDANN, the standard deviation of the average NN intervals calculated over short periods, usually 5 minutes, which is an estimate of the changes in heart rate due to cycles longer than 5 minutes; 2) the SDNN index (SDNNI) the mean of the 5-minute standard deviations of NN intervals calculated over 24 hours, which measures the variability due to cycles shorter than 5 minutes; and 3) RMSSD, the square root of the mean squared differences of successive NN intervals. Frequency domain parameters include low-frequency (LF) and high-frequency (HF) components. The measurement of LF and HF power components is usually made in absolute values of power (milliseconds squared). LF and HF may also be measured in normalized units, which represent the relative value of each power component in proportion to the total power minus the very-low-frequency component. The representation of LF and HF in normalized units emphasizes the controlled and balanced behavior of the two branches of the autonomic nervous system. Moreover, the normalization tends to minimize the effect of the changes in total power on the values of LF and HF components. Heart rate variability can be measured with spontaneous and controlled-rate breathing. The latter approach minimizes the effect of respiration on HRV.

While controlled human exposure data on acute cardiovascular responses to PM exposure have been accumulating due to directed research funding to support the epidemiological evidence of a PM-mortality association, the available data regarding the cardiovascular effects of O<sub>3</sub> are limited. Early controlled human exposure studies found little evidence of an O<sub>3</sub> effect on heart rate (Superko et al. 1984; Drechsler-Parks 1987).

Drechsler-Parks et al. measured cardiac output by the noninvasive impedance cardiography method after exposure to 0.60 ppm NO<sub>2</sub>, 0.45 ppm O<sub>3</sub>, and 0.60 ppm NO<sub>2</sub> and 0.45 ppm O<sub>3</sub> for 2 hours. The exercise-induced increase in cardiac output after NO<sub>2</sub>/O<sub>3</sub> exposure was significantly smaller than after exposure to filtered air or O<sub>3</sub> alone (Drechsler-Parks et al 1995). Gong et al measured cardiovascular physiological parameters in 6 healthy and 10 hypertensive volunteers exposed to 0.3 ppm O<sub>3</sub> for 3 hours (Gong et al. 1998). Ozone appeared to increase heart-rate blood pressure product and impaired pulmonary gas exchange. Limitations of this study include a small sample size and different ages for the healthy and hypertensive subjects making the comparison between these groups difficult.

Several groups have assessed the effects of exposure to combinations of PM and O<sub>3</sub>. In a series of studies, Brook and colleagues have exposed healthy volunteers for 2 hours to 0.12 ppm O<sub>3</sub> and 150 µg/m<sup>3</sup> concentrated ambient particles (CAPS) in Toronto and found that this exposure increased brachial artery constriction in one experiment and increased diastolic blood pressure in another compared to filtered air (FA) control (Brook et al. 2002; Urch et al. 2005). The most recent report from this group compared responses [blood pressure (BP), HRV, brachial flow-mediated dilatation (FMD), and several biomarkers] of healthy subjects exposed for 2 hours to O<sub>3</sub> alone, CAPS alone, the combination of the two, and FA; the experiments were conducted with different sets of subjects at two locations, Toronto and Ann Arbor, MI (Brook et al. 2009). No effects attributable to O<sub>3</sub> were reported. Another study conducted by our group with CARB funding reported decreased HRV after exposure to 0.2 ppm O<sub>3</sub> and 250 µg/m<sup>3</sup> carbon and ammonium nitrate particles, but not after exposure to the particles alone (Power et al. 2008). Two limitations of this study were the small sample size (n=5) and lack of an O<sub>3</sub> only exposure. A more recent combined CAPs and O<sub>3</sub> exposure study (122 µg /m<sup>3</sup> and 0.14 ppm, respectively) with a much larger sample size (n=59) showed a statistically significant enhancement of a particle effect by O<sub>3</sub> for the low-frequency component of HRV and approached significance for the high-frequency component and time-domain measures of HRV (Fakhri et al. 2009). Exposure to CAPs and O<sub>3</sub> also induced a significant increase in diastolic BP in this study.

From this summary, it should be apparent that additional and better data on cardiovascular responses to O<sub>3</sub> are needed. To address this data gap, we designed a study to address the question of whether short-term exposures to ambient levels of O<sub>3</sub> cause acute cardiovascular responses as assessed by HRV and systemic biomarkers of inflammation and coagulability. In addition, we were interested in determining whether any of these responses are correlated with O<sub>3</sub>-induced local lung injury/inflammation. Because asthmatic individuals have greater airway inflammation after exposure to O<sub>3</sub> than healthy individuals, we wished to compare the HRV and systemic biomarker responses of these two groups.

The specific objectives of the study were as follows: 1) to determine the effect of a low and high concentration of O<sub>3</sub> on HRV in healthy and asthmatic humans; 2) to determine the effect of low and high concentrations of O<sub>3</sub> on markers of airway and systemic inflammation, and coagulability in healthy and asthmatic humans; and 3) to investigate relationships between changes in HRV and changes in markers of airway and systemic inflammation in healthy and asthmatic humans.

## Materials and Methods

**Study Design:** This study had a repeated measure design in which subjects were exposed to either clean filtered air (FA), 100 ppb ozone (low dose), or 200 ppb ozone (high dose) for 4 hours in a climate-controlled chamber followed by bronchoscopy approximately 20 hours later. Cardiovascular, pulmonary, and hemostatic endpoints were measured immediately before exposure (0-hr), immediately after exposure (4-hr), and on the following morning prior to bronchoscopy (24-hr). Each subject returned and underwent all three exposure types with a minimum of 2 weeks in between exposure sessions to allow for recovery from any inflammation or injury sustained during the prior session. The order of exposures was counterbalanced and randomized.

**Subjects:** Twenty-five subjects were recruited via advertisements placed in University of California San Francisco (UCSF) campus newsletters, local San Francisco newspapers, and internet websites (e.g., [www.craigslist.org](http://www.craigslist.org)). Potential subjects were screened by health questionnaire, brief physical examination, spirometry, and exercise testing. The inclusion/exclusion criteria included: (1) age between 18 to 50 years; (2) ability to perform moderate-intensity exercise; (3) being healthy with no history of cardiovascular, hematologic, or pulmonary diseases other than mild asthma; (4) no history of acute infection within the past 6 weeks prior to start of the study; (5) non-smoker as defined by having a history of less than ½ pack-year lifetime tobacco use and no history of any tobacco use in the past 6 months; and (6) no history of illicit drug use. Asthmatic subjects were asked to stop their asthma and allergy medications in a sequential manner based on the duration of action of each medication (inhaled corticosteroids for 2 weeks, anti-histamines and leukotriene inhibitors for 3 days, long-acting bronchodilators for 2 days, and short-acting bronchodilators for 8 hr). The subjects were informed of the risks of the experimental protocol and signed a consent form that had been approved by the UCSF Committee on Human Research. All subjects received financial compensation for their participation.

After a telephone interview, subjects were scheduled for an initial visit to the laboratory, where a medical history questionnaire was completed. A 30-min exercise test designed to determine a workload that generated the target ventilatory rate was also completed on the initial visit.

**Climate-Controlled Chamber and Atmospheric Monitoring:** The experiment took place in a ventilated, climate-controlled chamber at 20°C and 50% relative humidity. The chamber is a stainless steel-and-glass room of 2.5 × 2.5 × 2.4 m (Model W00327-3R; Nor-Lake, Hudson, WI) that was custom-built and designed to maintain temperature and relative humidity within 2.0°C and 4% from the set points, respectively (WebCtrl Software; Automated Logic Corporation, Kennesaw, GA). Temperature and relative humidity were recorded every 30 s and displayed in real-time (LabView 6.1; National Instruments, Austin, TX).

**Exposure Session:** Each exposure session was 4 h long, with subjects exercising for the first 30 min and then resting for the following 30 min of each hour in the climate-controlled chamber. The exercise consisted of running on a treadmill or pedaling a cycle ergometer. Exercise intensity was adjusted for each subject to achieve a target expired minute ventilation (VE) of 20 L/min/m<sup>2</sup> body surface area. During exercise, VE was calculated (LabView 6.1; National Instruments, Austin, TX) from tidal volume and breathing frequency measured using a pneumotachograph at the 10-min and 20-min intervals of each 30-min exercise period. Subjects remained inside the chamber for the entire 4-h exposure period. The type of exposure (FA, low ozone, or high ozone) was chosen randomly prior to each session and was not revealed to the subjects. Immediately before (0-h), immediately after (4-h), and 20 h after (24-h) the exposure

period, subjects underwent phlebotomy, spirometry, blood pressure and heart rate measurement, and electrocardiographic (ECG) monitoring for HRV measurement.

*Spirometry:* Each subject's spirometry and peak expiratory flow were measured at each of the 0-h, 4-h, and 24-h time points. Spirometry was performed on a dry rolling-seal spirometer (PDS, nSpire Health, Inc., Longmont, CO) following American Thoracic Society performance criteria (Standardization of Spirometry, 1995). The best values for FVC and FEV1 from three acceptable FVC maneuvers were used in data analysis.

*Blood Sampling and Analysis:* A small amount of peripheral venous blood (30-40 ml) was withdrawn from the subject's arm at each of the 0-hr, 4-hr, and 24-hr time points. Blood was collected in appropriate sterile polypropylene tubes and processed by the UCSF clinical laboratory at San Francisco General Hospital or in our laboratory. Complete blood count with platelets (CBC) was performed using a light scatter flow cell method (Siemens ADVIA 2120; Deerfield, IL). Serum angiotensin-converting enzyme (ACE) activity level was measured using an enzymatic activity assay by ARUP Laboratories (Salt Lake City, UT). High-sensitive C-reactive protein (CRP) was measured via a latex enhanced immunoturbidometric method (Siemens ADVIA 1800).

Concentrations of serum cytokines at 0-hr and 4-hr were measured using a Milliplex human 14-plex cytokine assay (Millipore Corporation, St. Charles, MO). Cytokines measured included the following: granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 12 (IL-12 p70), monocyte chemotactic protein (MCP-1), and tumor necrosis factor alpha (TNF- $\alpha$ ). The lower limit of detection for GM-CSF, IL-1 $\beta$ , IL-5, IL-6, IL-7, IL-12 p70, MCP-1, and TNF- $\alpha$  was 3.2 pg/ml and for IL-8 was 16.0 pg/ml.

*Blood Pressure, Heart Rate, and HRV Measurement:* Blood pressure, heart rate, and HRV were also measured at 0-hr, 4-hr, 24-hr time points. The subjects were placed in a supine position on a gurney for 10 min to achieve a steady state and then underwent blood pressure and heart rate measurement using an automated sphyngomanometer (Critikon Model 8100; Tampa, FL). Subsequently, the subjects underwent ECG recording for a minimum of 10 min with a minimum of 5 min of spontaneous breathing followed by a minimum of 5 min of timed breathing using a metronome (12 breaths per min). Electrocardiograms were recorded using a Holter monitor (Forest Medical, LLC; East Syracuse, NY) connected to five electrodes placed in standard positions on the subject's chest wall to allow for the recording of two ECG lead channels.

Each ECG recording was then downloaded and coded. Spontaneous and metronome breathing periods were separated into two ECG files. These were then edited using Trillium 3000 software (Forest Medical, LLC; East Syracuse, NY). A single blinded investigator (KP) reviewed every recording for poorly defined and mislabeled beats. The Trillium 3000 software performed the HRV analysis using only normal beat-to-beat intervals. Each normal beat-to-beat interval was defined as the distance between the R-waves of two consecutive normal heartbeats on the ECG. Time domain variables were calculated using standard statistical methods described in the 1996 Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology paper as described previously (Task Force 1996). Frequency domain variables were calculated on each ECG using power spectral analysis. A clear 5-min epoch was defined from each ECG for this analysis. As the epochs used for analysis were time-defined, rather than beat-defined, discrete Fourier transform was used for power spectral analysis; with no windowing. The power spectrum was described by the total power and by three frequency components, very low frequency (VLF), low frequency (LF), and high frequency (HF). Additionally, LF and HF were

normalized with respect to the total power and the VLF [ $LF/(\text{total power-VLF}) \times 100$  and  $HF/(\text{total power-VLF}) \times 100$  respectively], and the LF to HF ratios (LF/HF) were derived.

*Bronchoscopy and Lavage Procedures:* Bronchoscopies were performed  $20 \pm 2$  h after exposure. This time was chosen because previous studies have documented the presence of an ozone-induced inflammatory response in many subjects at this time point (see sources from Arjomandi 2005a, 2005b). Our laboratory's procedures of bronchoscopy and BAL have been previously discussed in detail (Arjomandi 2005a, 2005b). Briefly, intravenous access was established, supplemental  $O_2$  was delivered, and the upper airways were anesthetized with topical lidocaine. Sedation with intravenous midazolam and fentanyl was used as needed for subject comfort. The bronchoscope was introduced through the mouth and vocal cords into the airways. The bronchoscope was then directed into the right middle lobe where lavage was performed with two 50-ml aliquots of 0.9 % saline warmed to  $37^\circ C$ . The fluid returned was designated BAL and immediately put on ice. After bronchoscopy, each subject was observed for an approximate 2-h recovery period. Total cells were counted on uncentrifuged aliquots of BAL using a hemocytometer. Differential cell counts were obtained from slides prepared using a cytocentrifuge, 25 g for 5 min, and stained with Diff-Quik as previously described (Arjomandi 2005a, 2005b). Cells were counted by two independent observers; the average of the two counts was used in data analysis. Bronchoalveolar lavage fluid was then centrifuged at 180 g for 15 min, and the supernatant was separated and re-centrifuged at 1,200 g for 15 min to remove any cellular debris prior to freezing at  $-80^\circ C$ .

*Data Management and Statistical Analysis:* All data were entered into a database (Microsoft Excel 2003; Microsoft; Redmond, WA) and then analyzed using STATA statistics software (STATA IE, version 10.0; StataCorp; College Station, TX). Each subject served as their own control. Data are presented as mean $\pm$ SD. A p-value of 0.05 was considered to be statistically significant in all analyses. All analyte concentrations were adjusted to account for possible changes in blood volume after exposure from the corresponding 0-h measurement in accordance with previous studies (Dill 1984; Harrison 1985; Rowbottom and Green 2000; Ahmadizad 2005). All concentrations were log-transformed before analysis, and their distributions were examined. Student's t-test was used for initial pair-wise comparisons. The change in a particular variable over the course of each exposure was calculated linearly using the 0-h value as the baseline. Because we noted on initial data inspection that exercise alone (i.e., the FA exposure condition) seemed to cause effects as great or greater than either concentration of  $O_3$ , we also analyzed FA data for non-bronchoscopy variables across the three time points (0-h, 4-h, and 24-h). To determine the presence of an  $O_3$  exposure-dependent response, a population-averaged generalized linear model was calculated.

## Results

*Subject Characteristics:* Subject characteristics for the exercise study are shown in Table 1. Of the 25 subjects enrolled in the study, 10 had mild asthma. Data for FA exposures were available for all 25, but only 22 completed both O<sub>3</sub> exposures. Of the 22 subjects with complete exposure data, seven had mild asthma (see Table 2). The subjects with asthma had a significantly mean lower FEV<sub>1</sub>. For the results reported below, n=25 for exercise-induced changes and n=22 for O<sub>3</sub>-induced changes. It should be noted that some of the standard deviations for results reported below are wide. This may be due to the relatively small sample size and the fact that both non-asthmatic and asthmatic subjects were enrolled.

*Climate-Controlled Chamber Conditions:* The average temperature and relative humidity in the climate-controlled chamber were (mean ± SD) 17.4 ± 1.1 °C and 54 ± 2.1%, respectively. The targeted O<sub>3</sub> concentrations were achieved. The mean concentration for each exposure was as follows: FA exposure, 14 ppb; low O<sub>3</sub> exposure, 107 ppb; and high O<sub>3</sub> exposure, 205 ppb.

*Exercise-induced Changes in Peripheral Blood Leukocytes:* The concentrations of peripheral blood leukocytes are shown in Table 3. The concentration of total leukocytes significantly increased at 4-h and remained elevated at 24-h (mean increase: 3.1±2.11×10<sup>6</sup> cells/ml, p<0.0001 and 0.85±0.9×10<sup>6</sup> cells/ml, p=0.0001, respectively) compared to 0-h. The concentrations of neutrophils, lymphocytes, and monocytes increased significantly at 4-h, and while the neutrophil concentration returned to baseline by 24-h, the concentrations of lymphocytes and monocytes remained elevated.

*Ozone-induced Changes in Peripheral Blood Leukocytes:* The concentrations of various peripheral blood leukocyte subtypes at each time point are shown in Table 4. While the total leukocytes increased at the 4-h time point and remained elevated at 24 h, there was no association between O<sub>3</sub> exposure and the change in leukocyte concentrations from 0 to 4 h (p = 0.93) and from 0 to 24 h (p = 0.45), suggesting that changes in leukocytes were mediated by exercise rather than O<sub>3</sub>. Similarly, we observed no association between O<sub>3</sub> exposure and the degree of change in total monocyte, neutrophil, and lymphocyte concentrations from 0 to 4 h and from 0 to 24 h.

Eosinophil concentrations decreased significantly from 0 to 4 h during low and high O<sub>3</sub> exposures; there was also a decrease during FA exposure, but it did not reach significance. Using linear regression models, we observed an association between the degree of eosinophil decrease from 0 to 4 h and O<sub>3</sub> exposure (parameter estimate ± SEM = -1.8 ± 0.6 ×10<sup>4</sup> cells/ml per 100 ppb O<sub>3</sub>; p = 0.004), suggesting that this decrease in eosinophils is due to at least in part to O<sub>3</sub>. This association remained significant (p = 0.005) after adjustment for presence of asthma. Given the non-significant effect seen with FA, O<sub>3</sub> may have enhanced a mild exercise-induced effect.

*Exercise-induced Changes in Peripheral Blood CRP, ACE, and Cytokines:* The concentrations of serum CRP and ACE activity are shown in Table 3. No significant change in CRP level occurred between 0-h and 4-h. However, at 24-h, CRP had significantly increased in the entire group (mean increase: 1.66±2.9 mg/L, p=0.011). Serum ACE activity levels showed a slight but significant increase at 4-h (mean decrease: 1.8±4.18 U/L, p=0.04) but returned back to baseline by 24-h.

MCP increased significantly from a baseline value of 542.4±54.5pg/ml to 726.2±81.9 pg/ml, and IL-6 from 4.6±1.3 pg/ml to 5.8±1.2pg/ml at 4-h (p=0.009 and p=0.03, respectively). No significant changes were observed for any other measured cytokines.

*Ozone-Induced Changes in Peripheral Blood CRP, ACE, and Cytokines:* Serum CRP concentration did not change at 4-h but increased at 24-h compared to baseline during all three exposure types (Table 4). The change in serum CRP from 0-h to 24-h across 200 ppb O<sub>3</sub> exposure was significantly higher than that across filtered air exposure (0.78±0.76 mg/L higher increase in mean CRP; p=0.01) (Figure 1). In linear regression analyses, we observed a trend towards an association between increasing O<sub>3</sub> exposure and the degree of change in CRP concentration between 0 to 24 h (parameter estimate ± SEM = 0.06 ± 0.04 for log-transformed CRP [mg/L] per 100 ppb O<sub>3</sub>; p = 0.09). Serum ACE activity levels were not significantly affected by O<sub>3</sub> exposure. No significant changes in cytokine levels were associated with O<sub>3</sub> exposure.

*Exercise-induced Changes in Peripheral Blood Coagulatory Indices:* Plasma fibrinogen did not change after exercise at any time point. In contrast, plasminogen activator inhibitor-1 (PAI-1) level decreased significantly at 4-h (mean decrease: 11.8±19.2IU/ml, p=0.005) but returned back to baseline level by 24-h. The changes in prothrombin time (PT) and partial thromboplastin time (PTT), though significant, were small in magnitude (Table 5).

*Ozone-induced Changes in Peripheral Blood Coagulatory Indices:* No association between increasing O<sub>3</sub> exposure and the degree of change in fibrinogen, PAI-1, PT, or platelets between 0 to 4 h and 0 to 24 h was observed (Table 6). PTT decreased at 4-h during the FA exposure, but not during either low or high ozone exposure.

*Ozone-induced Changes in BAL Inflammatory Indices:* Bronchoalveolar lavage data are shown in Table 7. Exposure to 200 ppb O<sub>3</sub> caused increased airway injury as evidenced by increased BAL total protein concentration compared to FA. Using linear regression models, we observed a trend towards an association between total protein concentration and O<sub>3</sub> exposure (parameter estimate ± SEM = 0.059 ± 0.035 µg/ml per 100 ppb ozone, p = 0.09). The concentrations of IL-6 and IL-8 were also linearly associated with O<sub>3</sub> exposure (log-transformed model parameter estimate ± SEM for IL-6 = 0.217 ± 0.096 pg/ml; p = 0.023; for IL-8 = 0.217 ± 0.082 pg/ml; p = 0.008), supportive of increased O<sub>3</sub>-induced expression of these cytokines. The association between the concentration of GM-CSF and O<sub>3</sub> exposure reached trend significance (log-transformed model parameter estimate ± SEM = 0.129 ± 0.066 pg/ml per 100 ppb O<sub>3</sub>; p = 0.0503). No significant associations were observed for IL-1β, IL-5, IL-7, IL-12, or TNF-α. Adjustment for total protein did not significantly change these results.

Bronchoalveolar lavage neutrophil and eosinophil concentrations increased with O<sub>3</sub> exposure (parameter estimate ± SEM for BAL neutrophils = 4.76 ± 2.00 ×10<sup>3</sup> cells/ml per 100 ppb O<sub>3</sub>; p = 0.019; for BAL eosinophils = 9.08 ± .41 ×10<sup>2</sup> cells/ml; p = 0.028). These associations remained significant after adjustment for the presence of asthma. Macrophage and lymphocyte concentrations in BAL did not change significantly with O<sub>3</sub> exposure.

*Ozone-Induced Changes in Lung Function:* Spirometric data are shown in Table 8. FEV<sub>1</sub> and FVC decreased at 4-h compared to baseline after 200 ppb O<sub>3</sub> exposure (a 5% mean percent decrease compared to baseline) but not after 100 ppb O<sub>3</sub> or FA exposure. A linear association between the degree of decrease in FEV<sub>1</sub> or FVC at 4-h and O<sub>3</sub> exposure was observed (parameter estimate ± SEM for FEV<sub>1</sub> = -0.074 ± 0.019 L per 100 ppb O<sub>3</sub>; p = 0.0002; for FVC = -0.061 ± 0.02 L per 100 ppb O<sub>3</sub>; p = 0.003). The FEV<sub>1</sub>/FVC ratio decreased at 4 h after 200 ppb O<sub>3</sub> exposure. This decrease was also linearly associated with O<sub>3</sub> exposure (parameter estimate ± SEM = -0.659 ± 0.32 per

100 ppb O<sub>3</sub>; p = 0.04). No significant differences in lung function due to O<sub>3</sub> exposure were observed at 24-h compared to baseline.

*Exercise-induced Changes in Cardiac Autonomic Function:* No significant changes in the standard deviation of all R-R intervals (SDNN), the standard deviation of the mean of all intervals (SDANN), or the root mean square of successive differences among normal R-R intervals (RMSSD) were observed for spontaneous breathing (Table 9). A significant decrease in the mean of all R-R standard deviations (SDNNI) at 4-h was observed for all subjects (mean decrease: 8.16±19.4 ms, p=0.046).

When the subjects underwent timed (metronome) breathing, RMSSD significantly decreased at 4-h (mean decrease: 10.3±21.9 ms, p=0.027). No significant changes were observed in RMSSD at 24-h or for any other index of HRV during timed breathing.

Analysis of the normalized frequency domain variables (LF, HF, LF/HF ratio) during spontaneous breathing did not reveal any significant changes across the exercise session (from 0-h to 4-h) (Table 10).

*Ozone-Induced Changes in HRV Frequency-Domain Indices:* Analysis of spontaneous-breathing HRV frequency-domain variables (LF, HF, LF/HF ratio) revealed that exposure to 200 ppb O<sub>3</sub> caused a 0 to 4-h increase in the normalized LF (and a corresponding decrease in the normalized HF) compared to the effect of exercise alone (p = 0.002) (Table 11). With 100 ppb O<sub>3</sub> exposure, these changes reached trend significance (p = 0.076). Exposure to 200 ppb O<sub>3</sub> also caused an increase in normalized LF, and corresponding decrease in normalized HF, from 0 to 24-h compared to the effect of exercise alone (p = 0.016) (Figure 2). A linear association between O<sub>3</sub> exposure and the 0 to 24-h change in LF (and corresponding change in HF) reached trend significance (parameter estimate ± SEM = 4.386 ± 2.239 per 100 ppb O<sub>3</sub>; p = 0.050). Ozone did not cause any significant change in frequency-domain HRV indices when subjects underwent timed breathing, although the direction of changes in these parameters was consistent with the values observed with spontaneous breathing.

*Correlations Between Other Biomarkers of O<sub>3</sub> Effects and HRV Frequency-Domain Indices:* Analysis of blood and BAL markers of O<sub>3</sub>-induced inflammation with the significant changes in normalized LF and HF showed no significant correlations. The O<sub>3</sub>-induced change in FEV<sub>1</sub>, however, did show a trend towards a positive correlation with the increase in LF from 0 to 24-h after exposure to 200 ppb (r= -0.18; p= 0.149).

## Discussion

In this research project, we first looked at the effects of intermittent moderate-intensity exercise as frequently used in human inhalational exposure studies on a variety of endpoints of relevance to cardiovascular responses to O<sub>3</sub> exposure. We found that exercise induced a systemic pro-inflammatory response characterized by an immediate post-exercise increase in peripheral blood monocyte, neutrophil, and lymphocyte counts; an immediate increase in serum IL-6 and MCP-1 concentrations; and a delayed increase in serum CRP at 24 h post-exercise. No substantial exercise-induced effects on coagulatory parameters were found. Furthermore, while significant decreases in SDNNI during spontaneous breathing and RMSSD during timed breathing were observed, the overall lack of significant changes in the majority of HRV indices suggests that intermittent moderate-intensity exercise does not considerably alter autonomic modulation of the heart if measured 30 min after the end of exercise. Together, these results indicate that the typical intermittent moderate-intensity exercise protocols used in inhalational exposure studies can cause an acute systemic pro-inflammatory response but no substantial coagulatory or cardiac autonomic changes.

After evaluating the effects of exercise on our endpoints of interest, we then proceeded to assess the effects of two levels of exposure to O<sub>3</sub>, a “low” exposure (100 ppb) and a “high” exposure (200 ppb), on these endpoints. As expected from previous research in our laboratory, the high O<sub>3</sub> exposure did induce a decrease in lung function, airway injury as manifested by an increase in total BAL protein, and airway inflammation as evidenced by increases in BAL neutrophils, eosinophils, and several pro-inflammatory cytokines. The low exposure caused no decrease in lung function and only mild airway inflammation. There was little evidence of a systemic inflammatory response to O<sub>3</sub>, with the exception of a trend toward an increase in serum CRP at 24 h after exposure. Moreover, no evidence of a pro-coagulatory response to O<sub>3</sub> exposure was found. Despite this lack of a systemic response, we did find that exposure to 200 ppb O<sub>3</sub> induced a significant increase in the normalized LF domain of HRV at both 4 and 24 h after exposure. This finding supports the results of our previous pilot study with five subjects in which a similar 4-h exposure to 200 ppb O<sub>3</sub> combined with carbon and ammonium nitrate particles induced an overall decrease in HRV. This finding is also consistent with a recent report on the immediate effects of a 2-h exposure to a higher level of O<sub>3</sub> (300ppb) on the high frequency domain of HRV (Devlin, Duncan et al. 2012). Moreover, our results extend these findings by demonstrating that the adverse changes in HRV are present at lower levels of exposure, may be dose-dependent, and persist at least up to 20h after the end of exposure.

### *Exercise effects*

Inhalational exposure studies commonly use intermittent exercise regimens in order to increase minute ventilation ( $V_E$ ) and maximize the “effective dose” of exposure to pollutants while decreasing total exposure time (Silverman et al., 1976, Adams et al., 1981). However, when studying the systemic inflammatory, coagulatory, and cardiac autonomic effects of inhaled pollutants, exercise may hamper the ability to measure pollutant-induced changes by decreasing the signal-to-noise ratio. Our study is the first to demonstrate that an exercise protocol used in inhalational exposure studies can significantly alter several markers of systemic inflammation (Donde et al. 2012). The repeated-measure cross-over design used in many of inhalational exposure studies may control for the confounding effects of exercise; however, a small pollutant signal may be difficult to detect against a background of significant exercise effect.

In our study, intermittent moderate-intensity exercise resulted in a post-exercise peripheral blood leukocytosis which was sustained at the 24-h time-point. While the initial leukocytosis was characterized predominantly by neutrophilia, the sustained leukocytosis was due to increases in monocyte and lymphocyte counts. Previous studies, using other exercise protocols, have also reported an immediate post-exercise peripheral blood neutrophilia. Nieman et al reported a post-exercise neutrophilia in 12 women who walked briskly for 45 min on a treadmill (average  $V_E$  of 37L/min) (Nieman et al., 1991). Meyer et al also found a similar immediate post-exercise neutrophilia in male subjects after repeated (up to eight times) bouts of maximal-intensity cycling for 60s (Meyer et al., 2001). On the other hand, reports on the effect of exercise on peripheral blood lymphocyte count have been mixed, with some studies reporting transient increases (Nieman et al., 1991, Nieman et al., 1995) and others reporting lymphocytopenia in the hours after exercise (Shinkai et al., 1992, Moyna et al., 1996). The variability in results may be due to variability in the intensity or duration of exercise protocols and/or selection of time-points for differential leukocyte counts. Our study provides a reference against which the effects of inhaled pollutants can be compared in inhalational exposure studies using intermittent moderate-intensity exercise.

In contrast to the systemic inflammation we observed with our exercise protocol, we did not find it to substantially affect peripheral blood coagulatory and fibrinolytic markers. Although peripheral blood PAI-1 activity in our subjects did significantly decrease post-exercise, this decrease may be due to diurnal variation rather than to a systemic coagulatory response to exercise given that PAI-1 is known to be higher in early morning hours (Angleton et al., 1989). A review of previous studies suggests that type and intensity of exercise have significant effects on whether or not any coagulatory or fibrinolytic changes are observed. A study with a similar exercise intensity to our own found no significant change in fibrinogen after a 30-min brisk walk in females (Davis et al., 2008), but another study found a transient increase in fibrinogen following a single 30-min session of high intensity resistance exercise (Ahmadizad and El-Sayed, 2005). Others have suggested that a minimum exercise intensity threshold must be reached to observe fibrinogen degradation products (Dufaux et al., 1991). Overall, we conclude that intermittent moderate-intensity exercise does not cause substantial coagulatory changes.

Our study also provides data that indicate that changes in HRV observed in controlled human exposure studies of pollutants involving exercise protocols are less likely to be due exercise than the pollutant of interest.

### *Ozone effects*

With our two levels of exposure to  $O_3$ , no evidence of an additional peripheral blood leukocytosis was observed beyond that seen with exposure to FA. However, in a linear exposure-response regression, there was a non-significant trend toward an additional effect of  $O_3$  exposure on the exercise-induced increase in CRP at 24 h after exposure. C-reactive protein is a non-specific biomarker of inflammation and this evidence of a possible effect of  $O_3$  is consistent with a mild systemic inflammatory response. The observed increase in CRP is in and of itself not likely to have any clinical relevance. Further investigation of the potential of  $O_3$  to induce a systemic inflammatory response is warranted.

While we did not find clear evidence of an  $O_3$ -induced systemic inflammatory response even at 200 ppb, this level of exposure was sufficient to cause airway injury and inflammation as evidenced by BAL leukocytosis and increased total protein and selected cytokine concentrations. Thus, a relatively high level of exposure to  $O_3$  that

caused a local lung inflammatory reaction did not lead to a clear systemic response. In addition, at the lower level of O<sub>3</sub> exposure, 100 ppb, there was suggestive evidence of a mild airway inflammatory response based on BAL neutrophil and IL-6 concentrations, consistent with previous studies from other laboratories.

The addition of O<sub>3</sub> to the protocol at either level of exposure appeared to have no effect on the coagulatory and fibrinolytic endpoints we measured. Thus, it is unlikely that exposure to ambient levels of O<sub>3</sub> is likely to lead to clinically relevant thrombotic events.

The significant increases in the LF domain of HRV that we observed after exposure to 200 ppb of O<sub>3</sub> compared to filtered air at both the 4-h and 24-h time points are not likely to be confounded by exercise. Additional support for O<sub>3</sub> induction of decreased HRV comes from the fact that we also observed non-significant increases in the LF domain with 100 ppb exposure at both the 4-h and 24-h time points. The presence of an exposure response-relationship when the LF data at all concentrations and time points were analyzed in a linear regression also supports the role of O<sub>3</sub> exposure in the observed decrease in LF HRV.

No previous controlled human exposure study has investigated the effect of O<sub>3</sub> on HRV. Even though we did not observe an overall decrease in HRV, the directional changes seen in the normalized frequency-domain variables are consistent with an effect of O<sub>3</sub>. The HF component of HRV is related to respiratory sinus arrhythmia (as mediated through the baroreflex) and is a marker of parasympathetic influence on heart rate (vagal tone), whereas the LF component is a marker for both sympathetic and parasympathetic modulation (Pagani et al. 1986). The HF and LF variables were normalized because large shifts in total power and VLF can mask smaller shifts in HF and LF (i.e., when the total power drops, the HF will drop also, although the proportion of HF relative to the total power may actually increase). Therefore, normalized frequency values better reflect the changes in sympathetic and parasympathetic tone (Task Force 1996). In our study, the normalized LF value, which is the best reflection of sympathetic tone, increased after the O<sub>3</sub> exposure as compared with FA exposure, whereas the normalized HF value, which is the best reflection of parasympathetic tone, decreased. Additionally, in our study, the LF-to-HF ratio increased after the O<sub>3</sub> exposure when compared with FA exposure, again indicating increased relative sympathetic tone (Pagani et al. 1986). These directional changes in normalized frequency domains are similar to what we observed in our previously published pilot study (Power et al. 2008).

The mechanism underlying our primary finding, that exposure to O<sub>3</sub> caused a significant increase in the normalized LF domain of HRV compared to exercise alone, remains to be elucidated. It may be related to the irritant effects of O<sub>3</sub> in the airways and increased autonomic responses to this nociceptive stimulation. However, the O<sub>3</sub> effect on HRV was present after both 100 and 200 ppb exposures, but only the higher exposure caused any effect on lung function, which is known to be mediated by neural pathways.

Our study investigated several potential mechanisms by which O<sub>3</sub> may lead to acute cardiovascular morbidity and mortality, including systemic inflammation, induction of a pro-coagulatory state, and autonomic stimulation. Although we found evidence only for an O<sub>3</sub> effect on autonomic pathways, we only assessed a limited set of biomarkers of systemic inflammation and coagulability. We did not measure adhesion molecules or von Willebrand factor, for example. We also did not investigate an oxidative stress pathway. Because O<sub>3</sub> is a prototypic oxidant gas that is known to cause lipid peroxidation through generation of reactive oxygen species in the airways, it is plausible that its adverse cardiovascular effects are mediated through oxidation of lipids. In a previous study, we did show an O<sub>3</sub>-induced increase in peripheral blood 8-isoprostane, a marker of lipid peroxidation and oxidative stress. Another potential adverse effect of O<sub>3</sub>-

induced oxidative stress that we did not investigate is endothelial dysfunction from impairment of NO metabolism.

The clinical relevance of any of the O<sub>3</sub>-induced effects that we observed is unclear. The mildly elevated CRP at 24 h is suggestive of a possible mild systemic inflammatory response, but no other indices of systemic inflammation that we measured showed significant changes. The increased variability in the LF domain is also an isolated finding of unclear clinical relevance. Perhaps our only findings with some clinical meaning are the well known lung function decrements and airway inflammatory changes observed after exposure to 200 ppb O<sub>3</sub>.

The available data regarding the cardiovascular effects of O<sub>3</sub> are extremely limited. Gong et al measured cardiovascular physiological parameters in 6 healthy and 10 hypertensive volunteers exposed to 0.3 ppm O<sub>3</sub> for 3 hours (Gong et al. 1998). Ozone appeared to increase heart-rate blood pressure product and impaired pulmonary gas exchange. Limitations of this study include a small sample size and different ages for the healthy and hypertensive subjects making the comparison between these groups difficult.

Several groups have assessed the effects of exposure to combinations of PM and O<sub>3</sub>. In a series of studies, Brook and colleagues have exposed healthy volunteers for 2 hours to 0.12 ppm O<sub>3</sub> and 150 µg/m<sup>3</sup> concentrated ambient particles (CAPS) in Toronto and found that this exposure increased brachial artery constriction in one experiment and increased diastolic blood pressure in another compared to filtered air (FA) control (Brook et al. 2002; Urch et al. 2005). The most recent report from this group compared responses [blood pressure (BP), HRV, brachial flow-mediated dilatation (FMD), and several biomarkers] of healthy subjects exposed for 2 hours to O<sub>3</sub> alone, CAPS alone, the combination of the two, and FA; the experiments were conducted with different sets of subjects at two locations, Toronto and Ann Arbor, MI (Brook et al. 2009). No effects attributable to O<sub>3</sub> were reported. Another study conducted by our group reported changes in HRV after exposure to 0.2 ppm O<sub>3</sub> and 250 µg/m<sup>3</sup> carbon and ammonium nitrate particles, but not after exposure to the particles alone (Power et al. 2008). Two limitations of this study were the small sample size (n=5) and lack of an O<sub>3</sub> only exposure (see below for more detailed information). The results of a recently published study of a combined O<sub>3</sub> and CAPS exposure with a much larger sample size (n=59) lend support to our findings that O<sub>3</sub> can enhance the effects of fine PM on HRV; O<sub>3</sub> also enhanced a PM effect on diastolic blood pressure (Fakhri et al. 2009).

Several studies published since we completed data collection provide conflicting evidence of the potential of O<sub>3</sub> to induce acute cardiovascular effects. In one study, 14 healthy subjects (age 22-47 years) underwent a 3-h exposure with intermittent exercise to either O<sub>3</sub> (250 ppb) or FA. Induced sputum was collected 3 h after exposure. Nineteen to 22 hours after exposure, ECG, blood pressure, cardiac output, and muscle sympathetic nerve activity were measured at rest and during various breathing maneuvers (Tank et al. 2011). While the O<sub>3</sub> exposure induced the expected airway inflammation, as indicated by a significant increase in sputum neutrophils, no significant effect on cardiovascular measurements was detected. In another field study of 20 healthy elderly subjects, ambient O<sub>3</sub> concentration was associated with decreased HRV. After adjusting for ambient PM<sub>2.5</sub> and NO<sub>2</sub>, the HF component changed -4.87% per 10 ppb increment of O<sub>3</sub>. In addition, there was a trend-significant decrease in the low LF component and a trend-significant increase in LF/HF (Jia et al. 2011). As noted above, a recent report by Devlin and colleagues showed effects on both the high frequency domain of HRV (immediately post-exposure) and systemic inflammation (24h post-exposure) with a 2-h exposure to 300ppb O<sub>3</sub> (Devlin, Duncan et al. 2012).

Our study has both strengths and limitations. The strengths include the following: an experimental study of the potential cardiovascular effects of O<sub>3</sub> in humans, a relatively large number of subjects for a controlled human exposure study, careful assessment of potentially confounding exercise effects, use of two different concentrations of O<sub>3</sub> that allowed an exposure-response analysis, and measurement of biomarkers of both systemic and airway inflammation.

Limitations include relative lack of power to study small changes (e.g., the trend toward an increase in CRP at 24 h after exposure might have become significant with a larger sample size) and study subjects that were relatively young and healthy. We chose to recruit such subjects for safety reasons, given that the effects of O<sub>3</sub> inhalation on cardiovascular endpoints in elderly subjects who might be at greater risk have not been previously studied. It is possible that only patients with pre-existing atherosclerotic cardiac disease are at risk for O<sub>3</sub>-induced acute effects (Jia et al. 2011). We chose to study asthmatic subjects who were otherwise healthy as a potentially susceptible subgroup because of pre-existing airway inflammation. We and others have shown that asthmatic subjects have greater airway inflammatory responses to O<sub>3</sub>, but this may not translate to any increased risk for cardiovascular morbidity. Another caveat is that in order to deliver a sufficient effective dose of O<sub>3</sub> capable of causing mild airway inflammation, all exposures were with intermittent exercise.

## Summary and Conclusions

The primary goal of this project was to determine whether short-term exposure to O<sub>3</sub> causes acute cardiovascular effects. Based on pilot data that was obtained in a previous CARB-supported study, we hypothesized that exposure to O<sub>3</sub> at ambient levels would lead to a decrease in heart rate variability (HRV), a parameter associated with increased risk of cardiac morbidity, and increased systemic inflammation and coagulability. Twenty five subjects (15 healthy and 10 asthmatic subjects) were recruited to participate in the study. The subjects were exposed to three conditions (filtered air, 100 ppb O<sub>3</sub>, and 200 ppb O<sub>3</sub>) in random order. Exposures were for 4 hours with intermittent exercise (30 min of each hour) at a minute ventilation of 20 L/kg/m<sup>2</sup> body surface area. Exercise is used in controlled human exposure studies to increase the effective dose of the pollutant. Heart rate variability was measured and blood samples were obtained immediately prior to, immediately after, and 20 h after each exposure period. Bronchoscopy to obtain bronchoalveolar lavage (BAL) fluid was performed at 20 h after exposure. The data analysis was done in two steps. First, the effect of exercise on HRV variability and biomarkers of systemic inflammation and coagulability was evaluated by comparing data across the filtered air exposure. Second, we investigated the effect of O<sub>3</sub> on HRV parameters and the biomarkers of interest, using the data collected at the two different concentrations to assess whether there was evidence of exposure-dependent responses.

We found that intermittent moderate-intensity exercise induced a pro-inflammatory systemic response characterized by increases in peripheral blood leukocyte counts, and C-reactive protein, monocyte chemotactic protein, and interleukin-6 concentrations. Exercise did not substantially alter levels of fibrinogen, angiotensin-converting enzyme, markers of coagulability, or HRV. We also found that exposure to 200 ppb O<sub>3</sub> induced a decrease in lung function, airway injury as manifested by an increase in total BAL protein, and airway inflammation as evidenced by increases in BAL neutrophils, eosinophils, and several pro-inflammatory cytokines. Exposure to 100 ppb O<sub>3</sub> caused no decrease in lung function and only mild airway inflammation.

Despite the induction of a local lung inflammatory response at 200 ppb, there was little evidence of a systemic inflammatory response to O<sub>3</sub>, with the exception of a trend toward an increase in serum CRP at 24 h after exposure. Moreover, no evidence of a pro-coagulatory response to O<sub>3</sub> exposure was found. Despite this lack of a systemic response, we did find that exposure to 200 ppb O<sub>3</sub> induced a significant increase in the normalized low frequency (LF) domain of HRV at both 4 and 24 h after exposure. We also observed non-significant increases in the LF domain with 100 ppb exposure at both the 4-h and 24-h time points. The presence of an exposure-relationship when the LF data at all concentrations and time points were analyzed in a linear regression also supports the role of O<sub>3</sub> exposure in the observed increase in LF HRV. To our knowledge this is the first controlled exposure study to find an effect on HRV with O<sub>3</sub> alone. The clinical significance of this effect is unclear.

## Recommendations

- 1) The finding that short-term exposure to O<sub>3</sub> induced an increase in the normalized low frequency domain of heart rate variability should be confirmed in another study. Because there appeared to be a non-significant effect of exposure to 100 ppb O<sub>3</sub> on this index of heart rate variability, future research should address ambient levels.
- 2) The roles of pulmonary and cardiovascular reflexes in acute responses to O<sub>3</sub> and perhaps PM<sub>2.5</sub> deserve further investigation.
- 3) Subjects with greater susceptibility to the acute cardiovascular effects of O<sub>3</sub> should be studied such as older individuals with pre-existing atherosclerosis.
- 4) The lack of systemic inflammatory or pro-coagulant responses to 200 ppb O<sub>3</sub> should also be confirmed.
- 5) Other pathways by which O<sub>3</sub> could cause acute adverse cardiovascular effects should be investigated, including oxidative stress-induced endothelial dysfunction.

## References

- Adams WC, Savin WM, Christo AE. 1981. Detection of ozone toxicity during continuous exercise via the effective dose concept. *J Appl Physiol* 51:415-422.
- Algra A, Tijssen JG, Roelandt JR, Pool J, Lubsen J. 1993. Heart rate variability from 24-hour electrocardiography and the 2-year risk for sudden death. *Circulation* 88:180-185.
- Ahmadizad S, El-Sayed MS: 2005. The acute effects of resistance exercise on the main determinants of blood rheology. *J Sports Sci* 23:243-249.
- Angleton P, Chandler WL, Schmer G. 1989. Diurnal variation of tissue-type plasminogen activator and its rapid inhibitor (PAI-1). *Circulation* 79:101-106.
- Arjomandi M, Schmidlin I, Girling P, Boylen K, Ferrando R, Balmes J. 2005a. Sputum induction and bronchoscopy for assessment of ozone-induced airway inflammation in asthma. *Chest* 128:416-423.
- Arjomandi M, Witten A, Abbritti E, Reintjes K, Schmidlin I, Zhai W, Solomon C, Balmes J. 2005b. Repeated exposure to ozone increases alveolar macrophage recruitment into asthmatic airways. *Am J Respir Crit Care Med* 172:427-432.
- Donde A, Wong H, Frelinger J, Power K, Balmes J, Arjomandi M. 2012. Effects of exercise on systemic inflammatory, coagulatory, and cardiac autonomic parameters in an inhalational exposure study. *J Occup Environ Med* 54:466-470.
- American Thoracic Society. 1995. Standardization of spirometry: 1994 update. *Am J Respir Crit Care Med* 152:1107-1136.
- Bigger JT, Jr., Fleiss JL, Steinman RC, Rolnitzky LM, Kleiger RE, Rottman JN. 1992. Frequency domain measures of heart period variability and mortality after myocardial infarction. *Circulation* 85:164-171.
- Brook RD. 2008. Cardiovascular effects of air pollution. *Clin Sci (Lond)* 115:175-187.
- Brook RD, Brook JR, Urch B, Vincent R, Rajagopalan S, Silverman F. 2002. Inhalation of fine particulate air pollution and ozone causes acute arterial vasoconstriction in healthy adults. *Circulation* 105:1534-1536.
- Brook RD, Urch B, Dvonch JT, Bard RL, Speck M, Keeler G, et al. 2009. Insights into the mechanisms and mediators of the effects of air pollution exposure on blood pressure and vascular function in healthy humans. *Hypertension* 54:659-667.
- Corradi M, Alinovi R, Goldoni M, Vettori M, Folesani G, Mozzoni P, et al. 2002. Biomarkers of oxidative stress after controlled human exposure to ozone. *Toxicol Lett* 134:219-225.
- Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. 2006. Biomarkers of oxidative damage in human disease. *Clin Chem* 52:601-623.

- deBoer RW, Karemaker JM, Strackee J. 1987. Hemodynamic fluctuations and baroreflex sensitivity in humans: a beat-to-beat model. *Am J Physiol* 253(3 Pt 2):H680-689.
- Dekker JM, Crow RS, Folsom AR, Hannan PJ, Liao D, Swenne CA, et al. 2000. Low heart rate variability in a 2-minute rhythm strip predicts risk of coronary heart disease and mortality from several causes: the ARIC Study. *Atherosclerosis Risk In Communities. Circulation* 102:1239-1244.
- Devlin RB, Duncan KE, Jardim M, Schmitt MT, Rappold AG, Diaz-Sanchez D. 2012. Controlled exposure of healthy young volunteers to ozone causes cardiovascular effects. *Circulation* 126:104-111.
- Dill DB, Costill DL. 1974. Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *J Appl Physiol* 37:247-248.
- Drechsler-Parks DM, Bedi JF, Horvath SM. 1987. Pulmonary function responses of older men and women to ozone exposure. *Exp Gerontol* 22:91-101.
- Drechsler-Parks DM. 1995. Cardiac output effects of O<sub>3</sub> and NO<sub>2</sub> exposure in healthy older adults. *Toxicol Ind Health* 11:99-109.
- Dufaux B, Order U, Liesen H. 1991. Effect of a short maximal physical exercise on coagulation, fibrinolysis, and complement system. *Int J Sports Med* 12 Suppl 1:S38-42.
- Fakhri AA, Ilic LM, Wellenius GA, Urch B, Silverman F, Gold DR, Mittleman MA. 2009. Autonomic effects of controlled fine particulate exposure in young healthy adults: effect modification by ozone. *Environ Health Perspect* 117:1287-92.
- Harrison MH. 1985. Effects on thermal stress and exercise on blood volume in humans. *Physiol Rev* 65:149-209.
- Hohnloser SH, Klingenhoben T, Zabel M, Li YG. 1997. Heart rate variability used as an arrhythmia risk stratifier after myocardial infarction. *Pacing Clin Electrophysiol* 20(10 Pt 2):2594-2601.
- Jia X, Song X, Shima M, Tamura K, Deng F, Guo X. 2011. Acute effect of ambient ozone on heart rate variability in healthy elderly subjects. *J Expo Sci Environ Epidemiol* 21:541-547.
- Kafoury RM, Pryor WA, Squadrito GL, Salgo MG, Zou X, Friedman M. 1999. Induction of inflammatory mediators in human airway epithelial cells by lipid ozonation products. *Am J Respir Crit Care Med* 160:1934-1942.
- Kaushal P, Taylor JA. 2002. Inter-relations among declines in arterial distensibility, baroreflex function and respiratory sinus arrhythmia. *J Am Coll Cardiol* 39:1524-1530.
- Liao D, Duan Y, Whitsel EA, Zheng ZJ, Heiss G, Chinchilli VM, et al. 2004. Association of higher levels of ambient criteria pollutants with impaired cardiac autonomic control: a population-based study. *Am J Epidemiol* 159:768-777.

- Lipsett MJ, Tsai FC, Roger L, Woo M, Ostro BD. 2006. Coarse particles and heart rate variability among older adults with coronary artery disease in the Coachella Valley, California. *Environ Health Perspect* 114:1215-1220.
- Martinon F. 2010. Signaling by ROS drives inflammasome activation. *Eur J Immunol* 40:616-619.
- Medinsky MA, Bond JA. 2001. Sites and mechanisms for uptake of gases and vapors in the respiratory tract. *Toxicology* 160:165-172.
- Moyna NM, Acker GR, Fulton JR, Weber K, Goss FL, Robertson RJ, Tollerud DJ, Rabin BS. 1996. Lymphocyte function and cytokine production during incremental exercise in active and sedentary males and females. *Int J Sports Med* 17:585-591.
- Nieman DC, Simandle S, Henson DA, Warren BJ, Suttles J, Davis JM, Buckley KS, Ahle JC, Butterworth DE, Fagoaga OR et al. 1995. Lymphocyte proliferative response to 2.5 hours of running. *Int J Sports Med* 16:404-409.
- Pagani M, Lombardi F, Guzzetti S, et al. 1986. Power spectral analysis of heart rate and arterial pressure variabilities as a marker of sympatho-vagal interaction in man and conscious dog. *Circ Res* 59:178-193.
- Pope CA, 3rd, Verrier RL, Lovett EG, Larson AC, Raizenne ME, Kanner RE, et al. 1999. Heart rate variability associated with particulate air pollution. *Am Heart J* 138:890-899.
- Pope CA, 3rd, Dockery DW. 2006. Health effects of fine particulate air pollution: lines that connect. *J Air Waste Manag Assoc* 56:709-742.
- Power KL, Balmes J, Solomon C. 2008. Controlled exposure to combined particles and ozone decreases heart rate variability. *J Occup Environ Med* 50:1253-1260.
- Pryor WA. 1994. Mechanisms of radical formation from reactions of ozone with target molecules in the lung. *Free Radic Biol Med* 17:451-465.
- Pryor WA, Squadrito GL, Friedman M. 1995. A new mechanism for the toxicity of ozone. *Toxicol Lett* 82-83:287-293.
- Rizvi AA. 2009. Cytokine biomarkers, endothelial inflammation, and atherosclerosis in the metabolic syndrome: emerging concepts. *Am J Med Sci* 338:310-318.
- Rowbottom DG, Green KJ. 2000. Acute exercise effects on the immune system. *Med Sci Sports Exerc* 32(7 Suppl):S396-405.
- Shinkai S, Shore S, Shek PN, Shephard RJ. 1992. Acute exercise and immune function. Relationship between lymphocyte activity and changes in subset counts. *Int J Sports Med* 13:452-461.

- Silverman F, Folinsbee LJ, Barnard J, Shephard RJ. 1976. Pulmonary function changes in ozone-interaction of concentration and ventilation. *J Appl Physiol* 41:859-864.
- Superko HR, Adams WC, Daly PW. 1984. Effects of ozone inhalation during exercise in selected patients with heart disease. *Am J Med*;77:463-470.
- Tank J, Biller H, Heusser K, Holz O, Diedrich A, Framke T, Koch A, Grosshennig A, Koch W, Krug N, Jordan J, Hohlfeld JM. 2011. Effect of acute ozone induced airway inflammation on human sympathetic nerve traffic: a randomized, placebo controlled, crossover study. *PLoS One* 6:e18737.
- Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. Special report: heart rate variability standards of measurement, physiological interpretation, and clinical use. *Circulation*. 1996;93:104–106.
- Urch B, Silverman F, Corey P, Brook JR, Lukic KZ, Rajagopalan S, et al. 2005. Acute blood pressure responses in healthy adults during controlled air pollution exposures. *Environ Health Perspect* 113:1052-1055.
- Zhang K. 2010. Integration of ER stress, oxidative stress and the inflammatory response in health and disease. *Int J Clin Exp Med* 3:33-40.

## Figure Legends

Figure 1: The concentration of serum CRP at 0-h, 4-h, and 24-h time points across filtered air (0ppb) (light gray), 100ppb (medium gray), and 200ppb (dark gray) O<sub>3</sub> exposure. The box represents the interquartile range; the horizontal line inside the box represents the median; and whiskers represent the minimum and maximum non-outlier values. Outlier values are shown by x.

Figure 2: The change in low and high frequency domain of HRV from 0-h to 4-h and 0-h to 24-h time points across filtered air (0ppb) (light gray), 100ppb (medium gray), and 200ppb (dark gray) O<sub>3</sub> exposure. The box represents the interquartile range; the horizontal line inside the box represents the median; and the whiskers represent the minimum and maximum non-outlier values. Outlier values are shown by x.

## Tables

**Table 1.** Subject characteristics: exercise study (n=25). Data presented as mean  $\pm$  SD.

<b>Subject Characteristics</b>	<b>All Subjects (n=25)</b>	<b>Female (n=14)</b>	<b>Male (n=11)</b>
Age (years)	33.0 $\pm$ 7.4	32.6 $\pm$ 8.3	33.3 $\pm$ 6.6
Height (cm)	169.4 $\pm$ 9.45	163.6 $\pm$ 6.1	176 $\pm$ 7.6
BMI (kg/m <sup>2</sup> )	26.1 $\pm$ 6.5	26.6 $\pm$ 8.0	25.6 $\pm$ 4.1
BSA (m <sup>2</sup> )	1.87 $\pm$ 0.28	1.69 $\pm$ 0.31	1.93 $\pm$ 0.17
V <sub>E</sub> (L/min/m <sup>2</sup> BSA)	19.98 $\pm$ 0.46	19.9 $\pm$ 0.27	20.1 $\pm$ 0.64
Mild Asthmatics [N(%)]	10 (40%)	5 (36%)	5 (45%)

BMI: body mass index; BSA: body surface area; V<sub>E</sub>: Minute Ventilation. p-value is for comparison between females and males.

**Table 2.** Subject characteristics: ozone study (n=22). Data presented as mean  $\pm$  SD.

<b>Subject Characteristics</b>	<b>All Subjects</b>	<b>Non-asthmatic</b>	<b>Asthmatic</b>	<b>p-value</b>
Number	22	15	7	
Age (years)	32.2 $\pm$ 7.6	31.5 $\pm$ 6.3	33.7 $\pm$ 10.1	0.54
FEV <sub>1</sub> (L)	3.4 $\pm$ 0.8	3.6 $\pm$ 0.8	3.1 $\pm$ 0.6	0.14
FEV <sub>1</sub> (% predicted)	96.5 $\pm$ 14.8	100.9 $\pm$ 15.1	87.1 $\pm$ 9.3	<b>0.03</b>
FVC (L)	4.4 $\pm$ 1.1	4.3 $\pm$ 1.1	4.6 $\pm$ 1.1	0.66
FVC (% predicted)	99.5 $\pm$ 13.7	100.5 $\pm$ 14.9	97.4 $\pm$ 11.3	0.63

FEV<sub>1</sub>: forced expiratory volume in 1 second; FVC: forced vital capacity.

**Table 3.** Exercise-induced changes in peripheral blood leukocytes, C-reactive protein (CRP), angiotensin-converting enzyme activity (ACE), and cytokines (monocyte chemoattractant protein [MCP] and interleukin-6 [IL-6]). Data presented as mean  $\pm$  SD.

Inflammatory Indices	All Subjects (n=25)		
	0-h	4-h	24-h
Leukocytes ( $\times 10^6$ cells/ml)	5.8 $\pm$ 1.6	<b>8.9 <math>\pm</math> 2.6 ***</b>	<b>6.6 <math>\pm</math> 1.9 ***</b>
Neutrophil ( $\times 10^4$ cells/ml)	350 $\pm$ 131	<b>621 <math>\pm</math> 233***</b>	361 $\pm$ 145
Lymphocyte ( $\times 10^4$ cells/ml)	162 $\pm$ 48	<b>191 <math>\pm</math> 59 ***</b>	<b>222 <math>\pm</math> 58 ***</b>
Monocyte ( $\times 10^4$ cells/ml)	30.0 $\pm$ 8.5	<b>41.3 <math>\pm</math> 14 ***</b>	<b>38.3 <math>\pm</math> 14.5 **</b>
Eosinophil ( $\times 10^4$ cells/ml)	22.3 $\pm$ 16	<b>19.8 <math>\pm</math> 15.5 *</b>	23.4 $\pm$ 13.9
CRP (mg/L)	2.64 $\pm$ 4.7	2.77 $\pm$ 4.8	<b>4.30 <math>\pm</math> 5.3 **</b>
ACE (U/L)	40.1 $\pm$ 14.6	<b>41.9 <math>\pm</math> 15.4 *</b>	41.5 $\pm$ 14.6
MCP (pg/ml)	542 $\pm$ 54.5	<b>726 <math>\pm</math> 81.9 **</b>	ND
IL-6 (pg/ml)	4.6 $\pm$ 1.3	<b>5.8 <math>\pm</math> 1.2 *</b>	ND

Statistically significant data are shown in bold. \* 0.05 $\geq$ p>0.01; \*\* 0.01 $\geq$ p>0.001; \*\*\* p $\leq$ 0.001 (vs. the corresponding 0-h).

ND = not done

**Table 4.** Ozone-induced changes in peripheral blood leukocytes, C-reactive protein (CRP), and angiotensin-converting enzyme activity (ACE). Data presented as mean±SD.

Blood Inflammatory Indices	Filtered Air (0 ppb)			Low Ozone (100 ppb)			High Ozone (200 ppb)		
	0 hr	4 hr	24 hr	0 hr	4 hr	24 hr	0 hr	4 hr	24 hr
Leukocytes (×10 <sup>6</sup> cells/ml)	5.53 ± 1.23 <sup>a,b</sup>	8.69 ± 2.60 <sup>a</sup>	6.47 ± 1.74 <sup>b</sup>	5.93 ± 1.28 <sup>d,e</sup>	9.22 ± 2.16 <sup>d</sup>	6.45 ± 1.32 <sup>e</sup>	5.75 ± 1.25 <sup>g,h</sup>	8.95 ± 2.69 <sup>g</sup>	6.49 ± 1.36 <sup>h</sup>
Neutrophils (×10 <sup>4</sup> cells/ml)	329.41 ± 109.83 <sup>a</sup>	608.85 ± 241.05 <sup>a</sup>	346.89 ± 131.53	359.88 ± 100.03 <sup>d</sup>	650.98 ± 182.78 <sup>d</sup>	347.68 ± 95.66	338.79 ± 95.10 <sup>g</sup>	641.17 ± 258.15 <sup>g</sup>	351.05 ± 120.61
Lymphocytes (×10 <sup>4</sup> cells/ml)	158.93 ± 43.98 <sup>a,b</sup>	186.07 ± 47.35 <sup>a</sup>	220.56 ± 54.33 <sup>b</sup>	163.35 ± 48.65 <sup>d,e</sup>	195.09 ± 60.54 <sup>d</sup>	206.94 ± 59.20 <sup>e</sup>	168.32 ± 40.24 <sup>h</sup>	181.97 ± 54.14	217.27 ± 51.54 <sup>h</sup>
Monocytes (×10 <sup>4</sup> cells/ml)	29.91 ± 8.28 <sup>a,b</sup>	40.63 ± 11.89 <sup>a</sup>	38.61 ± 14.75 <sup>b</sup>	34.12 ± 10.91 <sup>d,e</sup>	44.29 ± 14.02 <sup>d</sup>	38.05.84 <sup>e</sup>	31.77 ± 11.00 <sup>g,h</sup>	41.35 ± 12.14 <sup>g</sup>	37.29 ± 12.88 <sup>h</sup>
Eosinophils (×10 <sup>4</sup> cells/ml)	20.32 ± 15.09	17.90 ± 13.52	21.70 ± 13.78	21.07 ± 14.75 <sup>d</sup>	17.32 ± 12.21 <sup>d</sup>	23.60 ± 14.04	21.25 ± 14.14 <sup>g</sup>	15.22 ± 10.83 <sup>g</sup>	23.18 ± 15.85
CRP (mg/L)	1.68 ± 2.20 <sup>b</sup>	1.80 ± 2.41	3.54 ± 4.00 <sup>b</sup>	2.04 ± 3.79 <sup>e</sup>	2.15 ± 4.16	4.06 ± 4.87 <sup>e</sup>	2.30 ± 3.21 <sup>h</sup>	2.24 ± 3.05	4.98 ± 5.23 <sup>h</sup>
ACE (U/L)	40.36 ± 15.59 <sup>a</sup>	42.63 ± 16.38 <sup>a</sup>	41.75 ± 15.55	40.41 ± 16.43	40.98 ± 16.41	41.17 ± 16.43	39.14 ± 13.83	38.66 ± 12.90	39.93 ± 13.25

a, b, d, e, g, h: The letters indicate pair-wise comparisons with significant differences (p<0.05).

**Table 5.** Peripheral blood coagulatory changes with exercise. Data presented as mean  $\pm$  SD.

Coagulatory Indices	All Subjects (n=25)		
	0-h	4-h	24-h
Fibrinogen (mg/dL)	311 $\pm$ 117	307 $\pm$ 64	304 $\pm$ 93
PAI-1 (IU/ml)	13.8 $\pm$ 21.6	<b>2.1 <math>\pm</math> 4.3 **</b>	17.3 $\pm$ 29.8
Platelets ( $\times 10^6$ cells/ml)	289.6 $\pm$ 73.8	<b>303 <math>\pm</math> 73.3 **</b>	281.1 $\pm$ 70.4
PT (s)	13.1 $\pm$ 0.6	13.2 $\pm$ 0.7	<b>13.4 <math>\pm</math> 0.5 *</b>
PTT (s)	29.9 $\pm$ 3.5	<b>28.5 <math>\pm</math> 3.7 **</b>	<b>30.6 <math>\pm</math> 3.5 *</b>

Statistically significant data are presented in bold.

\*  $0.05 \geq p > 0.01$ ; \*\*  $0.01 \geq p > 0.001$ ;

PAI-1 Plasminogen activator-inhibitor 1 activity;

PT: Prothrombin time; PTT: Partial thromboplastin time.

**Table 6.** Peripheral blood coagulatory changes with ozone exposure. Data presented as mean  $\pm$  SD.

Coagulatory Indices	Filtered Air (0 ppb)			Low Ozone (100 ppb)			High Ozone (200 ppb)		
	0 hr	4 hr	24 hr	0 hr	4 hr	24 hr	0 hr	4 hr	24 hr
Fibrinogen (mg/dL)	314.43 $\pm$ 93.27	295.40 $\pm$ 48.61	304.39 $\pm$ 54.06	297.77 $\pm$ 53.46	299.89 $\pm$ 46.31	318.23 $\pm$ 60.27	312.81 $\pm$ 54.26	292.20 $\pm$ 52.55	317.21 $\pm$ 53.83
PAI-1 (IU/ml)	13.93 $\pm$ 22.04 <sup>a</sup>	3.62 $\pm$ 3.92 <sup>a</sup>	17.60 $\pm$ 31.34	12.68 $\pm$ 23.85	5.32 $\pm$ 11.18	19.86 $\pm$ 32.46	11.16 $\pm$ 18.77	9.53 $\pm$ 29.63	13.85 $\pm$ 20.93
Platelets ( $\times 10^6$ cells/ml)	290.59 $\pm$ 62.65 <sup>a,b</sup>	299.41 $\pm$ 63.97 <sup>a</sup>	268.18 $\pm$ 56.65 <sup>b</sup>	274 $\pm$ 55.66 <sup>d,e</sup>	283.77 $\pm$ 61.14 <sup>d</sup>	261.09 $\pm$ 65.56 <sup>e</sup>	284.95 $\pm$ 55.98 <sup>h</sup>	290.41 $\pm$ 53.50	267.04 $\pm$ 56.36 <sup>h</sup>
PT§ (s)	13.1 $\pm$ 0.60 <sup>b</sup>	13.21 $\pm$ 0.68	13.34 $\pm$ 0.63 <sup>b</sup>	13.02 $\pm$ 0.59 <sup>d</sup>	13.18 $\pm$ 0.55 <sup>d</sup>	13.21 $\pm$ 0.61	13.14 $\pm$ 0.52	13.13 $\pm$ 0.52	13.27 $\pm$ 0.53
PTT § (s)	30.04 $\pm$ 3.54 <sup>a</sup>	28.46 $\pm$ 3.89 <sup>a</sup>	30.59 $\pm$ 3.57	29.57 $\pm$ 2.88 <sup>d</sup>	28.25 $\pm$ 2.44 <sup>d</sup>	29.72 $\pm$ 2.78	29.725 $\pm$ 3.57	28.98 $\pm$ 3.21	30.09 $\pm$ 2.84

a, b, d, e, h: The letters indicate pair-wise comparisons with significant differences ( $p < 0.05$ ).  
 § N = 17 for filtered air, 17 for low ozone, and 16 for high ozone

**Table 7.** Ozone-induced airway inflammation. Data presented as mean  $\pm$  SD.

BAL Inflammatory Indices	Filtered Air (0 ppb)	Low Ozone (100 ppb)	High Ozone (200 ppb)
Total Protein ( $\mu\text{g/ml}$ )	71.95 $\pm$ 35.77 <sup>b</sup>	73.51 $\pm$ 41.08	103.32 $\pm$ 44.24 <sup>b</sup>
IL-1 $\beta$ (pg/ml)	0.147 $\pm$ 0.056	0.143 $\pm$ 0.055 <sup>c</sup>	0.151 $\pm$ 0.073 <sup>c</sup>
IL-5 (pg/ml)	0.753 $\pm$ 0.052 <sup>a</sup>	0.203 $\pm$ 0.231 <sup>a</sup>	0.36 $\pm$ 0.927
IL-6 (pg/ml)	2.35 $\pm$ 2.95 <sup>b</sup>	3.49 $\pm$ 4.38	4.60 $\pm$ 4.16 <sup>b</sup>
IL-7 (pg/ml)	0.671 $\pm$ 0.821	0.845 $\pm$ 0.627	1.19 $\pm$ 1.77
IL-8 (pg/ml)	11.62 $\pm$ 17.32 <sup>a,b</sup>	15.96 $\pm$ 13.55 <sup>a</sup>	14.51 $\pm$ 12.18 <sup>b</sup>
IL-12 (pg/ml)	0.173 $\pm$ 0.186	0.169 $\pm$ 0.165	0.149 $\pm$ 0.059
GM-CSF (pg/ml)	2.18 $\pm$ 2.11	2.20 $\pm$ 1.71	3.33 $\pm$ 2.23
TNF- $\alpha$ (pg/ml)	0.518 $\pm$ 1.12	0.401 $\pm$ 0.495	0.448 $\pm$ 0.331
BAL Total Leukocytes ( $\times 10^4$ cells/ml)	20.8 $\pm$ 11.8	21.7 $\pm$ 10.8	22.7 $\pm$ 10.6
BAL Macrophages ( $\times 10^4$ cells/ml)	19.5 $\pm$ 12.7	17.7 $\pm$ 10.2	18.0 $\pm$ 6.9
BAL Lymphocytes ( $\times 10^4$ cells/ml)	3.3 $\pm$ 2.4	2.7 $\pm$ 1.5	2.6 $\pm$ 2.1
BAL Neutrophils ( $\times 10^4$ cells/ml)	0.56 $\pm$ 0.56 <sup>b</sup>	0.98 $\pm$ 0.91	1.51 $\pm$ 1.79 <sup>b</sup>
BAL Eosinophils ( $\times 10^4$ cells/ml)	0.09 $\pm$ 0.09	0.19 $\pm$ 0.22	0.29 $\pm$ 0.39

a, b: The letters indicate pair-wise comparisons with significant differences ( $p < 0.05$ ).

**Table 8.** Ozone-induced changes in lung function. Data presented as mean  $\pm$  SD.

Pulmonary function Indices	Filtered Air (0 ppb)			Low Ozone (100 ppb)			High Ozone (200 ppb)		
	0 hr	4 hr	24 hr	0 hr	4 hr	24 hr	0 hr	4 hr	24 hr
FEV1 (L)	3.44 $\pm$ 0.78 <sup>b</sup>	3.42 $\pm$ 0.80	3.33 $\pm$ 0.80 <sup>b</sup>	3.32 $\pm$ 0.79 <sup>e</sup>	3.32 $\pm$ 0.83	3.27 $\pm$ 0.79 <sup>e</sup>	3.39 $\pm$ 0.8 <sup>g,h</sup>	3.22 $\pm$ 0.88 <sup>g</sup>	3.29 $\pm$ 0.77 <sup>h</sup>
FVC (L)	4.42 $\pm$ 1.10 <sup>b</sup>	4.39 $\pm$ 1.10	4.34 $\pm$ 1.09 <sup>b</sup>	4.34 $\pm$ 1.08	4.31 $\pm$ 1.10	4.31 $\pm$ 1.10	4.39 $\pm$ 1.1 <sup>g,h</sup>	4.24 $\pm$ 1.15 <sup>g</sup>	4.32 $\pm$ 1.09 <sup>h</sup>
FEV1/FVC ratio	0.79 $\pm$ 0.11 <sup>b</sup>	0.79 $\pm$ 0.10	0.78 $\pm$ 0.10 <sup>b</sup>	0.78 $\pm$ 0.11	0.78 $\pm$ 0.91	0.77 $\pm$ 0.11	0.78 $\pm$ 0.1 <sup>g,h</sup>	0.77 $\pm$ 0.10 <sup>g</sup>	0.77 $\pm$ 0.11 <sup>h</sup>

b, e, g, h: The letters indicate pair-wise comparisons with significant differences ( $p < 0.05$ ).

**Table 9.** Heart rate variability with exercise. Data presented as mean  $\pm$  SD.

HRV Time-Domain Indices (ms)	All Subjects (n=25)		
	0-h	4-h	24-h
SDNN-spontaneous	53.8 $\pm$ 25.8	53.0 $\pm$ 35.7	57.9 $\pm$ 27.3
SDNN-metronome	55.7 $\pm$ 26.5	51.4 $\pm$ 30.6	58.7 $\pm$ 24.1
SDANN-spontaneous	0.48 $\pm$ 2.2	3.4 $\pm$ 8.6	3.7 $\pm$ 7.4
SDANN-metronome	38.3 $\pm$ 31.7	33.2 $\pm$ 32.2	38.3 $\pm$ 31.7
SDNNI-spontaneous	52.1 $\pm$ 23.8	<b>44.0 <math>\pm</math> 28.9*</b>	51.4 $\pm$ 30.7
SDNNI-metronome	30.1 $\pm$ 15.3	30.2 $\pm$ 17.3	31.1 $\pm$ 11.0
RMSSD-spontaneous	43.4 $\pm$ 31.7	41.2 $\pm$ 43.2	45.1 $\pm$ 29.8
RMSSD-metronome	45.2 $\pm$ 31.4	<b>34.9 <math>\pm</math> 31.3*</b>	47.6 $\pm$ 31.0

Statistically significant data are shown in bold.

\* 0.05  $\geq$  p > 0.01 (vs. the corresponding 0-h);

SDNN: Standard deviation of all R-R intervals; SDANN: Standard deviation of the mean of all intervals; SDNNI: Mean of all R-R standard deviations; RMSSD: Root mean square of successive differences in normal R-R intervals.

**Table 10.** Heart rate variability frequency-domain indices with exercise. Data presented as mean  $\pm$  SD.

HRV Frequency-Domain Indices	All Subjects (n=25)	
	0-h	4-h
LFn (nu)	58.1 $\pm$ 16.7	56.2 $\pm$ 15.9
HFn (nu)	41.9 $\pm$ 16.7	43.8 $\pm$ 15.9
LF (ms <sup>2</sup> )	799.9 $\pm$ 1492.6	625.5 $\pm$ 1004.2
HF (ms <sup>2</sup> )	751.5 $\pm$ 1793.7	618.2 $\pm$ 1132.7
LF/HF ratio	1.81 $\pm$ 1.3	1.7 $\pm$ 1.3

LF: low frequency component; HF: high frequency component; LFn: normalized low frequency component; HFn: normalized high frequency component; nu: normalized unit.

**Table 11.** Heart rate variability frequency-domain indices with ozone exposure. Data presented as mean  $\pm$  SD.

HRV Indices	Filtered Air (0 ppb)			Low Ozone (100 ppb)			High Ozone (200 ppb)		
	0 hr	4 hr	24 hr	0 hr	4 hr	24 hr	0 hr	4 hr	24 hr
LF - spont (normalized units)	54.36 $\pm$ 18.60	58.45 $\pm$ 13.82	51.5 $\pm$ 17.95	49.10 $\pm$ 14.83	55.24 $\pm$ 16.10	52 $\pm$ 15.57	46.59 $\pm$ 16.17 <sup>g,h</sup>	55.32 $\pm$ 16.06 <sup>g</sup>	51.55 $\pm$ 15.60 <sup>h</sup>
HF - spont (normalized units)	45.64 $\pm$ 18.60	41.55 $\pm$ 13.82	48.5 $\pm$ 17.95	50.90 $\pm$ 14.83	44.76 $\pm$ 16.10	48 $\pm$ 15.57	53.41 $\pm$ 16.17 <sup>g,h</sup>	44.68 $\pm$ 16.06 <sup>g</sup>	48.45 $\pm$ 15.60 <sup>h</sup>
LF/HF Ratio - spont	1.71 $\pm$ 1.49	1.71 $\pm$ 1.05	1.45 $\pm$ 1.23	1.16 $\pm$ 0.78	1.54 $\pm$ 0.96	1.31 $\pm$ 0.78	1.06 $\pm$ 0.72 <sup>g,h</sup>	1.52 $\pm$ 0.91 <sup>g</sup>	1.28 $\pm$ 0.71 <sup>h</sup>
LF - metro	28.41 $\pm$ 17.18	37.27 $\pm$ 20.28	31.04 $\pm$ 17.96	29.05 $\pm$ 14.56	35.95 $\pm$ 17.11	29.85 $\pm$ 16.93	29.59 $\pm$ 13.98	33.59 $\pm$ 16.66	27.73 $\pm$ 15.45
HF - metro	71.59 $\pm$ 17.18 <sup>a</sup>	62.72 $\pm$ 20.28 <sup>a</sup>	68.95 $\pm$ 17.96	70.95 $\pm$ 14.56	64.05 $\pm$ 17.11	70.15 $\pm$ 16.93	70.41 $\pm$ 13.98	66.41 $\pm$ 16.66	72.27 $\pm$ 15.45
LF/HF Ratio - metro	0.50 $\pm$ 0.51	0.88 $\pm$ 1.08	0.58 $\pm$ 0.57	0.46 $\pm$ 0.65	0.71 $\pm$ 0.62	0.52 $\pm$ 0.43	0.48 $\pm$ 0.72	0.62 $\pm$ 0.47	0.47 $\pm$ 0.44

a, g, h: The letters indicate pair-wise comparisons with significant differences ( $p < 0.05$ ).

Figure 1

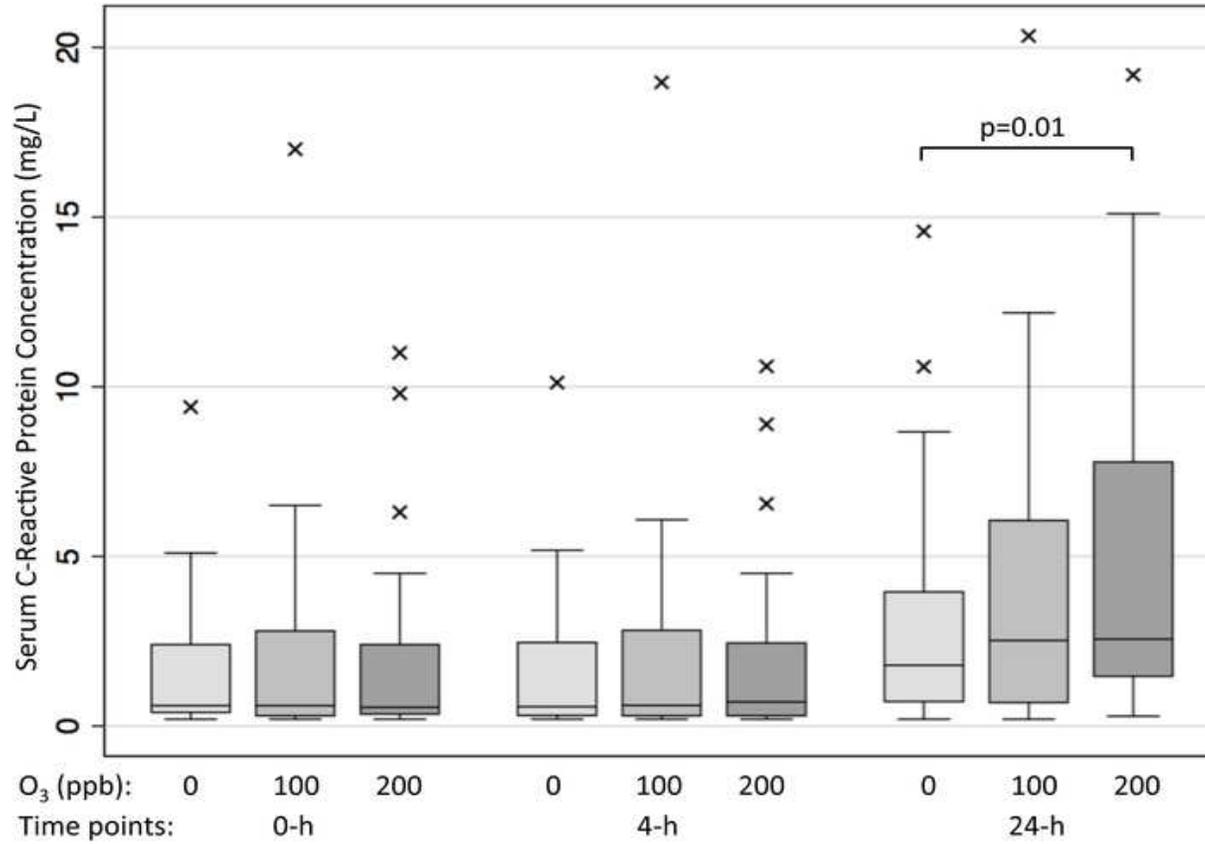
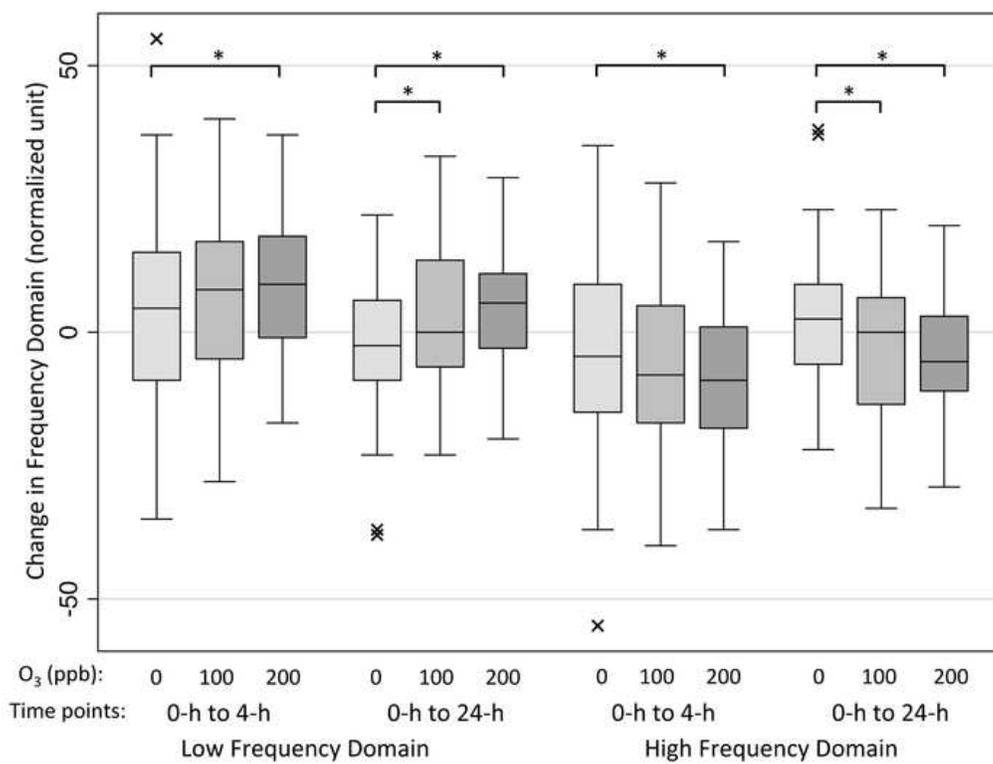


Figure 2



## Glossary of Terms, Abbreviations, and Symbols

ACE:	angiotensin converting enzyme
BAL:	bronchoalveolar lavage
CAPS:	concentrated ambient particles
CBC:	complete blood count
CRP:	C-reactive protein
ECG:	electrocardiogram
FA:	filtered air
FEV <sub>1</sub> :	forced expiratory volume in 1 second
FVC:	forced vital capacity
GM-CSF:	granulocyte macrophage colony-stimulating factor,
HF:	high-frequency component of HRV
HRV:	heart rate variability
IL-1 $\beta$ :	interleukin-1 $\beta$
IL-5:	interleukin 5
IL-6:	interleukin 6
IL-7:	interleukin 7
IL-8:	interleukin 8
IL-12 p70:	interleukin 12
LF:	low-frequency component of HRV
MCP-1:	monocyte chemotactic protein
NN:	normal-to-normal intervals (i.e., all intervals between adjacent QRS complexes resulting from sinus node depolarizations)
PM:	particulate matter
PM2.5:	particulate matter $\leq 2.5 \mu\text{m}$ in aerodynamic diameter
PT:	prothrombin time
PTT:	partial thromboplastin time
RMSSD:	the square root of the mean squared differences of successive NN intervals
ROS:	reactive oxygen species
RTLFL:	respiratory tract lining fluid
SDNN:	standard deviation of the NN intervals
SDANN:	the standard deviation of the average NN intervals calculated over short periods, usually 5 minutes, which is an estimate of the changes in heart rate due to cycles longer than 5 minutes
SDNNI:	the SDNN index, the mean of the 5-minute standard deviations of NN intervals calculated over 24 hours, which measures the variability due to cycles shorter than 5 minutes
TNF- $\alpha$ :	tumor necrosis factor-alpha
VE:	minute ventilation