Cardiorespiratory Effects of Ambient Particulates: Controlled Human Exposures to Concentrated Ambient $PM_{2.5}$ (CAP) Alone and With Added Ozone

by

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A thesis submitted in conformity with the requirements
For the degree Master of Science
Institute of Medical Sciences
University of Toronto

Acknowledgements

The author wishes to thank all those who assisted with this research, including:

The thesis committee: Frances Silverman, Susan Tarlo, and Jim Purdham for their expert guidance.

The laboratory team: Bruce Urch, Zdravko Lukic, Mary Rutherford, Jason Datema and Joanne Kubay for their excellent technical assistance and advice.

Those who collaborated on this project: J. Brook, B. Zimmerman, P. Corey, L. Liu, I. Broder, G. Tofler, E. Downar and R. Dales.

The technician who analyzed the Holter tapes for heart rate variability parameters: Mary Spilker, Harvard.

Those who funded this project: National Sanitorium Association; Environment Canada; Health Canada

And most of all, I thank my family and friends for their love, support and understanding during the research and writing of this manuscript.

The work presented in this thesis was a result of a collaboration of a multidisciplinary team. My contribution to this work was limited to: the background literature review; administration of sputum and nasal lavage tests; cardiovascular tests, including ECG and Holter hook-up; review of ECG and Holter tapes (from Study No. 2); laboratory analysis of sputum and nasal lavage samples (Study No. 2); interpretation of the data; and preparation of this document.

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Glossary of Terms

BS	black smoke (measurement parameter for particulates)
CAP	concentrated ambient particles smaller than 2.5 microns in aerodynamic diameter ($PM_{2.5}$)
CO	carbon monoxide
COH	coefficient of haze (measurement parameter for particulates)
ECG	electrocardiogram
FA	filtered air
FVC	forced vital capacity
HEF	human exposure facility
HF	high frequency
HRV	heart rate variability
LF	low frequency
$\mu g/m^3$	micrograms per cubic meter
ms	milliseconds
NO	nitric oxide
NO_2	nitrogen dioxide
O_3	ozone
PEF	peak expiratory flow
$PM_{2.5}$	ambient particles smaller than 2.5 microns in aerodynamic diameter
PM_{10}	ambient particles smaller than 10 microns in aerodynamic diameter
pNN50	proportion of cycles where the difference is more than 50 milliseconds
ppb	parts per billion
ppm	parts per million
rMSDD	square root of the mean squared differences of successive NN intervals
SDNN	standard deviation of normal-to-normal RR intervals
SO_2	sulfur dioxide
TSP	total suspended particulates

Abstract

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Epidemiological studies suggest that there may be adverse human health effects associated with exposure to ambient fine particles ($PM_{2.5}$). In preliminary studies, we examined the health effects of concentrated ambient $PM_{2.5}$ (CAP) in downtown Toronto using the Harvard ambient particle concentrator. In the initial study, four young, healthy volunteers were each exposed to filtered air (FA), and CAP levels ranging from 23 to 124 $\mu g/m^3$ for two hours. In the second study, four young, asthmatic individuals were exposed to FA, up to 130 $\mu g/m^3$ CAP and/or 80 ppb ozone. Response measures included inflammatory cells, blood coagulation factors and cardiac effects. Using the parameters examined in these pilot studies, we were not able to demonstrate that the exposure to up to 130 $\mu g/m^3$ CAP alone or with 80 ppb ozone has acute health effects in young healthy or asthmatic individuals. The statistical significance of this was not assessed due to the small number of subjects in these pilot safety studies. Additional research with more subjects will be required to further examine the cardiorespiratory effects of $PM_{2.5}$.

1.0 INTRODUCTION

1.1 Overview

Air pollutants have long been known to cause health problems in susceptible members of the population, with effects ranging from increased respiratory symptoms to increased mortality on high air pollution days. In the last decade, fine particles are one component of the complex mixture of pollutants that have been associated with adverse health effects in epidemiological studies. Currently there is a paucity of data regarding health effects following controlled exposures with ambient fine particles. This thesis was part of a larger study that examined the health effects of controlled exposures to "real-world" ambient fine particulate matter in preliminary safety studies with human subjects. This research was the first to examine health effects in controlled human exposure studies of exposure to a combination of ambient particles and another common pollutant, ozone. Health endpoints for the studies included pulmonary function, inflammatory changes in respiratory tract lining fluid, and cardiovascular effects. Effects on pulmonary function were monitored by other researchers, and will not be discussed in detail in this thesis.

1.1.1 Sources and Chemistry of Ambient Particulates

Ambient particles are complex, heterogeneous particles that originate from natural and anthropogenic sources. The chemical composition of particles depends on the source and varies with geographical location (Spurny, 1996). Particles in the air that are smaller than 10 μm in aerodynamic diameter (PM_{10}) include a fine particle and coarse particle fraction. Fine particles are those smaller than 2.5 μm in aerodynamic diameter ($PM_{2.5}$) and coarse particles are between 2.5 and 10 μm in aerodynamic diameter (Environment

Canada, 1997). The chemical composition of particulate matter varies spatially and temporally, depending on the sources of the particles, and there are differences in eastern and western North America with higher concentrations of sulfates due to coal burning in the east (Spengler *et al.*, 1996).

Coarse particles are largely formed by mechanical disruption of soils or dust suspension (e.g., fugitive dust emissions from soils that result from wind erosion) (Schwartz *et al.*, 1999). Chemical analyses of PM_{10} suggest that the coarse fraction is made up of many elements, including calcium (Ca), silicon (Si), sodium (Na), iron (Fe) and aluminum (Al), that are derived from natural sources such as soil or road dust (Environment Canada, 1997). Pollen, mold, road salt and sea salt are also found in the coarse fraction (Wilson and Suh, 1997).

The fine fraction is likely from combustion processes, with high concentrations of sulfates (SO_4^{2-}), ammonium ion (NH_4^+), nitrogen oxides (NO_x), metals and organic compounds. Anthropogenic sources for fine particles include vehicle emissions, residential heating and electrical power plants (Environment Canada, 1997). Fine particulate matter may be formed via several mechanisms, including direct emissions of primary particles from combustion processes and formation of secondary fine particles from condensation of vapour in the atmosphere (Wilson and Suh, 1997). Since gases are present in air as well as particles, health effects of gaseous pollutants are important in considering the toxicity of particulate matter.

Ambient air pollution in the Toronto area is largely due to transportation, industry, residential heat and power generation as well as long-range transport of fine particles

from the United States (US) (Burnett *et al.*, 1997a). In fact, southern Ontario has been referred to as the "transport region" (Spengler *et al.*, 1996), since it is downwind of emissions in the US. Fine particles can be suspended in air for weeks and drift for hundreds to thousands of kilometers across national boundaries, while coarse particles tend to settle in a matter of hours, traveling less than one to ten kilometers (Wilson and Suh, 1997). Additionally, fine particles readily penetrate buildings to indoor air (Brauer *et al.*, 1989) through sides of windows, doors, and fresh air intakes. Wallace (1996) estimated that particulate matter from outdoor air contributes approximately 65% of fine particles and 43% of coarse particles indoors based on air exchange rates. Therefore, people are exposed to ambient fine particles while indoors as well as outdoors. Indoor sources of particles will not be discussed as part of this thesis since the studies presented here focus on outdoor $PM_{2.5}$.

1.1.2 Ambient Concentrations of Particulates

In remote areas of North America, annual average background concentrations of outdoor particulate matter vary from 4 to 12 $\mu g/m^3$ PM_{10} and 1 to 5 $\mu g/m^3$ $PM_{2.5}$ (CEPA/FPAC, 1999a). Background levels of particulates vary with: i) season, due to altered concentrations of pollen, spores and humidity; ii) geographical location, due to windblown dust or coastal sea salt spray; and iii) distance to industrial areas, due to long-range transport of fine particles.

In a study of 24 communities in the US and Canada from 1988-1991, Spengler *et al.* (1996) showed that mean daily concentrations of $PM_{2.1}$ and PM_{10} were similar in the US

and Canada, ranging from 5.8 to 20.7 and 16.5 to 32.7 $\mu g/m^3$, respectively. In Canada, most monitoring sites have mean 24-hour PM_{10} and $PM_{2.5}$ concentrations of 28 and 14 $\mu g/m^3$, respectively (Brook *et al.*, 1997). Mean ambient air pollutant concentrations in Toronto averaged from 1980-1994 (Burnett *et al.*, 1999) are listed in Table 1-1. These ambient levels were considered when determining the levels of pollutants to be used in the studies presented in this thesis.

Table 1-1: Daily Air Pollutant Concentrations in Toronto

Pollutants	Units	Mean Concentration	5^{th} - 100^{th} Percentile
$PM_{2.5}$	($\mu g/m^3$)	18.0	8-90
$PM_{2.5-10}$	($\mu g/m^3$)	12.2	3-68
PM_{10}	($\mu g/m^3$)	30.2	14-116
CO	(ppm)	1.18	0.5-6.1
NO_2	(ppb)	25.2	13-82
SO_2	(ppb)	5.35	0-57
O_3	(ppb)	19.5	3-90

(Burnett *et al.*, 1999)

1.1.3 Relevance to Human Health

Particles in the PM_{10} fraction can be inhaled into the respiratory tract since they are not filtered out by cilia in the nose and can impact the lungs (e.g., bronchi) (CEPA/FPAC, 1999). The $PM_{2.5}$ fraction of particles in the air are able to penetrate to deeper portions of the lung (e.g., bronchioles and alveoli) (Pinkerton *et al.*, 1995; Environment Canada, 1997; US EPA, 1997).

The biological response of individuals to particles depends on deposition, retention and clearance of particulates in each individual (Utell and Drew, 1998). Deposition of

particles in humans depends on the size of the airways and pattern of branching of the lungs, the size and shape of the particle, breathing rate, tidal volume, flow rate and whether the person breathes through the mouth or nose (Bascom *et al.*, 1996; Utell and Drew, 1998; McClellan and Miller, 1997). People with chronic obstructive pulmonary disease (COPD) have increased fractional deposition ((inhaled minus exhaled)/inhaled) of fine particles in the lung than people with normal lung function (Bennett *et al.*, 1995; Kim and Kang, 1995). Additionally, exposure to particles is enhanced with increased exercise due to increased total lung deposition rate of fine particles (Kim *et al.*, 1999). Increased deposition of particles in the lungs may result in increased exposure, thereby increasing the potential for adverse health effects.

1.1.4 Regulatory Status

In epidemiological studies, increased levels of ambient particulate matter has been associated with increased hospital admissions in Toronto, Southern Ontario, and other Canadian cities (Thurston *et al.*, 1994; Burnett *et al.*, 1994, 1997c, 1999), and increased cardiovascular mortality in Toronto (Ozkanyak *et al.*, 1995). Based on the results of available studies, regulators in Canada and the US are currently assessing the health impacts of fine particles in air.

Regulation of air pollutants needs to be cost-effective and result in low health risks for the population. However, it is difficult to set regulatory levels to be risk-free for all people in society, due to the heterogeneous nature of our population, and the presence of

sick or elderly people who may be more prone to disease. (Lipfert, 1997). This has been indicated by the Canadian federal government, as follows: "The new framework recognizes that it is not feasible to protect all members of a receptor population from adverse effects of air pollution at all times" (CCME, 1999a).

In Canada, the reference levels provided by the Canadian Council of Ministers of the Environment (CCME) for $PM_{2.5}$ and PM_{10} are 15 and 25 $\mu g/m^3$, respectively (CCME, 1999a). These reference levels are for a 24-hour averaging time. There are no federal levels provided for a 1-hour average or an annual averaging time. The Canada Wide Standard for $PM_{2.5}$ is 30 $\mu g/m^3$ as a 24-hour averaging time by the year 2010 (CCME, 1999b). In Ontario and BC, the interim ambient air quality criterion for 24-hour average PM_{10} is 50 $\mu g/m^3$ (MOEE, 1997; Air Resources Branch, 1995). The annual US EPA PM_{10} standard is 50 $\mu g/m^3$, with a 24-hour standard of 150 $\mu g/m^3$. The US EPA (1997) has proposed National Ambient Air Quality Standards for $PM_{2.5}$ of 15 $\mu g/m^3$ annually and 65 $\mu g/m^3$ for a 24-hour period. Some debate exists over these values, since many researchers believe that there are currently insufficient scientific data available to make conclusions regarding the toxicity of ambient fine particulate matter (McClellan and Miller, 1997). At present, there is a great deal of research underway to resolve the knowledge gaps regarding health effects of particles in air, including the biological mechanisms of toxicity.

It is uncertain whether the mass, size or composition of the particles are important for toxicity (Reichhardt, 1996; Brook *et al.*, 1997). It has been speculated that the biological mechanisms of particle toxicity may be due to acidity, metal composition, or physical

properties of ultrafine particles. Smaller particles have a higher proportion of chemicals adsorbed to their surface than coarse particles based on mass due to the increased surface area (Brook *et al.*, 1997). Recent studies with dogs have found cardiac effects to be variable on different days with similar CAP mass concentrations, suggesting that particle composition is important for toxicity (J.J. Godleski, personal communication). However, the results of these studies are preliminary and could be due to natural variability in a small sample size.

Currently, a lot of research is being conducted to identify the health effects of exposure to ambient particles in air. Some of the relevant articles will be discussed in this thesis, although it should be noted that there are several recent reviews that summarize available research on ambient particulate matter. These include:

- R. Wilson and J. Spengler (eds). 1996. *Particles in Our Air. Concentrations and Health Effects.* Harvard University Press. Harvard School of Public Health.

- Vedal, S. 1995. Health effects of inhalable particles: Implications for British Columbia. Prepared for the Air Resources Branch, British Columbia Ministry of Environment, Lands and Parks;

- Vedal, S. 1996. Evaluation of health impacts due to fine inhalable particles ($PM_{2.5}$). Contract Report Prepared from Health Canada, final report. November 1996;

- CEPA/FPAC. 1999a. *National Ambient Air Quality Objectives for Particulate Matter. Part I, Science Assessment Document.* Canadian Environmental Protection Act;

- CEPA/FPAC. 1999b. *National Ambient Air Quality Objectives for Particulate Matter. Addendum to the Science Assessment Document.* Canadian Environmental Protection Act;

- Ontario Ministry of the Environment, Ontario Smog Plan Steering Committee. 1999. A Compendium of Current Knowledge on Fine Particulate Matter in Ontario. PIBS 3798e; and

- US EPA. 1999. Air Quality Criteria for Particulate Matter. US Environmental Protection Agency. EPA 600/P-99/002a,b,c.

The above documents also discuss the uncertainties and knowledge gaps associated with the available data and regulation of particles. Other documents have also been prepared that address the uncertainties in the current database regarding health effects of ambient particles, including:

- CRESTech/NERAM. 1999. Information Gaps and Uncertainties in the IP/RP Compendium Documents and their Impact on Strategic Options. Expert Panel Final Report. A Scoping Study administered by CRESTech/NERAM. Network for Environmental Risk Assessment and Management.

- Canada-Wide Standards for PM and Ozone. Record of Stakeholder input on the Second National Multi-Stakeholder Consultation Workshop. Calgary. May 26-28, 1999.

The above reviews have been prepared largely for regulators to summarize the copious amount of literature available regarding the potential health impacts of ambient particles. This is important since the data available are used to determine appropriate levels of ambient particles in air that are protective of the health of the general population. The

following sections of this thesis will provide a brief review of some recent literature in order to provide an overview for the audience. It is beyond the scope of this assessment to prepare a comprehensive review of all data available for this topic.

1.2 Epidemiology Studies

For many years, air pollution has been known to adversely affect human health, although there have been no major air pollution episodes since the 1970's (Utell and Drew, 1998). Although air quality has generally been improving in North America, recent epidemiological studies suggest that there may be adverse health effects associated with ambient air quality. Increases in ambient $PM_{2.5}$ have been associated with increases in morbidity and mortality in a number of epidemiology studies in Canada and other countries. Epidemiological studies have shown a wide range of effects from increases in respiratory symptoms and decreases in lung function to increases in cardiorespiratory hospitalization and mortality (US EPA, 1997). Many studies with particulate matter, including total suspended particulate matter (TSP), black smoke (BS), and PM_{10} have shown an increase in mortality at ambient levels of particulates, largely in sick and elderly members of the population. It is unclear whether the mortality is due to: i) a "harvesting" effect, where sick/elderly people die a few days earlier due to increased air pollution levels (Reichhardt, 1996); ii) long-term reduced lifespan of population (e.g., chronic effects of exposure to elevated ambient particulate levels); or iii) increased acute mortality in sensitive populations. No threshold "safe" level has been established for ambient particulate matter (US EPA, 1997; Lipfert, 1997) based on epidemiological studies.

1.2.1 $PM_{2.5}$

Decreased lung function, measured as forced vital capacity (FVC) and peak expiratory flow (PEF), of adult hikers at Mt. Washington, New Hampshire was associated with increases in ambient $PM_{2.5}$ and O_3 levels, particularly in people with a history of asthma or wheeze (Korrick *et al.*, 1998). In this area, mean $PM_{2.5}$ levels were 10 $\mu g/m^3$ with a maximum of 60 $\mu g/m^3$, and O_3 levels were 21-74 ppb. In another study with non-smoking asthmatic adults in Erfurt, East Germany, decreased PEF, increased cough and increased feeling of illness during the day were associated with both the fine and ultrafine particulate fractions (Peters *et al.*, 1997). Exposure levels ranged from 9.1 to 350 $\mu g/m^3$ $PM_{2.5}$. Peters *et al.* (1997) suggested that the decrease in PEF and increase in illness could be due to inflammation from particle deposition.

An increase in non-trauma mortality was associated with increased $PM_{2.5}$ levels in Philadelphia, with a 2% increase in mortality for each 10 $\mu g/m^3$ in previous day $PM_{2.5}$ levels (Dockery *et al.*, 1996a). There was also a positive association with ground level ozone (0.9% per 10 $\mu g/m^3$) that was independent and additive to $PM_{2.5}$ effects. The authors reported a weaker association for PM_{10}, no association with coarse particles, SO_2 or aerosol acidity, and a negative association with CO. Daily mortality in six US cities (Boston, Knoxville, St. Louis, Steubenville, Portage and Topeka) was more strongly associated with $PM_{2.5}$ than with PM_{10} or the coarse fraction of PM_{10} (Schwartz *et al.*, 1996a). There was a statistically significant association between two-day mean $PM_{2.5}$ increases of 10 $\mu g/m^3$ and total daily mortality (1.5%), COPD mortality (3.3%), and deaths from ischemic heart disease (2.1%) (Schwartz *et al.*, 1996a). The authors reported

smaller increases in mortality with PM_{10} and SO_4^{2-} and no association with coarse particle mass (2.5-10 μm) or aerosol acidity (H^+) concentrations. Schwartz *et al.* (1996a) observed effects at $PM_{2.5}$ levels below 25 μg/m^3, and it was suggested that if there is a threshold for $PM_{2.5}$, it is less than 25 μg/m^3. It has been suggested that the lack of threshold may be due to a number of things, including inadequate exposure assessments, inclusion of all members of the population, including the sick and infirm, or difficulties with the statistical modelling conducted (McClellan, 1997).

One parameter associated with increased cardiovascular mortality is decreased heart rate variability (HRV), which is a measure of autonomic nervous system activity. In a recent abstract, the HRV (*i.e.*, standard deviation of normal-to-normal RR intervals (SDNN)) of 21 adults aged 53-87 years was decreased in association with higher levels of ambient $PM_{2.5}$ (maximum hourly levels were 9-72 μg/m^3) (Gold *et al.*, 1998). The authors suggested there may be increased sympathetic and decreased vagal tone associated with exposure to ambient $PM_{2.5}$. In another study, Pope *et al.* (1999a) found particulate matter was associated with increased heart rate, decreased SDNN, and increased vagal activity as rMSDD (square root of the mean squared differences of successive NN intervals) in seven subjects. Another recent epidemiological study with 90 elderly subjects in Utah Valley found increases in ambient PM_{10} of 100 μg/m^3 were associated with a small increase in heart rate of 0.8 beats/min in elderly subjects (Pope *et al.*, 1999b).

Recent epidemiological studies of cardiorespiratory hospital admissions using multi-pollutant models have shown stronger associations with gaseous pollutants than with particulate matter in Toronto, Canada (Burnett *et al.*, 1997a, 1999). Ozone was more

strongly associated with cardiorespiratory hospital admissions than $PM_{2.5}$ or PM_{10}. The effect of gaseous pollutants on morbidity from multi-pollutant models is very important in the assessment of health effects of particulate matter, especially when evaluating results from single-pollutant models. More studies using multi-pollutant models are required to determine what the health effects are from particles versus other gaseous pollutants in the air.

1.2.2 Other Pollutants

More epidemiological studies are available for PM_{10} and other measures of particulate matter than $PM_{2.5}$, due to the availability of monitors and historical data. Total suspended particulates (TSP) include large particles in the air, up to 40 or 100 μm in diameter (CEPA/FPAC, 1999b). Coefficient of haze (COH) is an optical measure of particle concentrations and is associated with carbon particles, specifically combustion of diesel fuel. COH is indicative of small PM rather than large PM, although COH does not measure a specific size fraction of particles. Black smoke (BS) which refers to a method used to measure the density of black stain on filter paper after air is drawn through the paper, and is not as relevant to vehicle emissions since they are generally less black than historical industrial emissions. Some of the more recent studies with particles and other air pollutants are presented below.

Increases of 10 $\mu g/m^3$ PM_{10} were associated with respiratory illness and asthma in working adults in Anchorage, Alaska (Gordian *et al.*, 1996). In this study mean PM_{10} levels ranged from 45 to 145 $\mu g/m^3$. In Finland, increased levels of ambient PM_{10} and BS

were significantly associated with a decline in PEF of asthmatic school children (Pekkanen *et al.*, 1996, 1997). The study controlled for temperature, time trend, autocorrelation, weekend/holiday effects, NO_2, NO, and SO_2. It is interesting that this study did not find fine and ultrafine particle levels to be more strongly associated than PM_{10} and BS concentrations.

Fine particles from combustion sources may be very acidic in nature, and some scientists speculate that the acidic nature of the particles may play a part in the toxicity of particles. Children living in areas with high levels of 'particle strong acidity' (monitored by pH analysis) in 24 communities in the US and Canada had a significant increase in respiratory symptoms (i.e., bronchitis), when compared with children living in less polluted areas, but not asthma (Dockery *et al.*, 1996b). Particle strong acidity ranged from 1.9 to 8.5 nmol/m^3 in this area and $PM_{2.5}$ levels ranged from 5.8 to 20.7 $\mu g/m^3$. In the same communities, Raizenne *et al.* (1996) found decreased lung function, as measured by FVC and FEV_1, in children was associated with particle strong acidity.

In a recent study, Hiltermann *et al.* (1998) found that adults with intermittent to severe asthma had increased asthma exacerbations on days with increased ambient PM_{10} and O_3 levels. This included shortness of breath and bronchodilator use, but no associations with PEF. In this study, it was shown that the severity of the asthma was not important for a response to PM_{10} and O_3, with mild asthmatics just as likely to react as severe asthmatics.

Increased levels of PM_{10} and O_3 were associated with daily hospital admissions for respiratory disease in the elderly in Spokane, WA where the mean levels of PM_{10} and O_3

are 46 $\mu g/m^3$ and 56 ppb, respectively (Schwartz, 1996). Similar results were found in Cleveland where the mean levels of PM_{10} and O_3 are 43 $\mu g/m^3$ and 56 ppb, respectively (Schwartz *et al.*, 1996b). Additionally, PM_{10} and CO were significantly associated with hospital admissions for cardiovascular disease for the elderly in Tucson, Arizona (Schwartz, 1997) but not with SO_2, O_3 or NO_2. Similarly, in Detroit, there was a significant association between PM_{10} concentrations (mean of 48 $\mu g/m^3$) and hospital admissions for ischemic heart disease in the elderly (Schwartz and Morris, 1995). There was also increased risk associated with SO_2 and CO but not O_3. A threshold for effects was not seen in this study. Respiratory hospital admissions in Detroit were significantly associated with increases in PM_{10} and $PM_{2.5}$ (Ito *et al.*, 1998).

PM_{10} has been associated with daily mortality in a number of studies. In Utah County, there were positive associations with PM_{10} and total mortality and cardiorespiratory mortality, but no evidence of a threshold for effects (Pope and Kalkstein, 1996). In addition, increases of PM_{10} were associated with increased daily total mortality of the elderly in Sao Paulo, Brazil (Saldiva *et al.*, 1995) in a study that evaluated PM_{10}, NO_x, SO_2, and CO. Daily mean concentrations of PM_{10} were 82.4 $\mu g/m^3$. In Sydney, Australia where the annual average PM_{10} levels are 20 $\mu g/m^3$, daily mortality was associated with ambient levels of PM_{10}, O_3 and to a lesser extent with NO_2 (Morgan *et al.*, 1998).

In a study of 10 cities in Canada, including Toronto, positive associations for congestive heart failure hospital admissions in the elderly were found for CO, NO_2, SO_2 and COH (Burnett *et al.*, 1997b). Burnett *et al.* (1997b) found that CO was most strongly associated with the increase and there was no significant association with O_3. In another study of

Ontario towns and cities, including Toronto, increases of 13 $\mu g/m^3$ particulate sulfate levels were associated with increased cardiac and respiratory hospital admissions of 2.8 % and 3.7%, respectively (Burnett *et al.*, 1995). In an earlier study, respiratory hospital admissions in Southern Ontario were positively and statistically significantly associated with O_3 in the summer months and particulate sulfates (Burnett *et al.*, 1994). Another study of 16 Canadian cities also found increases in hospital admissions for respiratory disease was associated with increases in air pollutants. The strongest association for excess admissions for respiratory disease was found with O_3, and a positive association was also seen with COH and CO (Burnett *et al.*, 1997c).

Ozone is common gaseous pollutant that is known to cause adverse health effects in susceptible members of the population (Koenig, 1999). Most of the known health effects of ozone are associated with the respiratory tract. Some of the recent epidemiological studies have been discussed above. College students from regions with high ozone levels (more than 80 ppb) had increased respiratory symptoms (chronic phlegm and wheeze) and decreased lung function (FEV_1 and FEF_{25-75}) compared with students from areas with low ozone levels (less than 80 ppb) (Galizia and Kinney, 1999). In another study, on days when ozone was elevated, some hikers at Mt. Washington experienced decreased lung function, measured as FVC, FEV_1 and PEF for 50 ppb increases in O_3 (Korrick *et al.*, 1998). Increased cardiac and/or respiratory hospital admissions have been reported in a number of regions following elevated ozone episodes (Burnett *et al.*, 1997a,c, 1999; Moolgavkar *et al.*, 1997; Steib *et al.*, 1996). Some studies have also documented increased all-cause or cardiovascular mortality to be associated with elevated ambient ozone concentrations (Burnett *et al.*, 1998; Ponka *et al.*, 1998).

1.2.3 Summary of Epidemiological Studies

Although a large number of epidemiological studies have shown cardiorespiratory effects to be associated with increases in ambient particulate levels, there is controversy over the findings. While epidemiological studies regarding human health effects of particulates have shown statistical associations, they may not necessarily be indicative of causality (Moolgavkar and Leubeck, 1996). There are few $PM_{2.5}$ monitors used in the world to determine exposure estimates, and therefore, most of the information upon which regulatory standards could be established for $PM_{2.5}$ is from inferred associations with PM_{10} and TSP (McClellan and Miller, 1997). Ratios for the amount of $PM_{2.5}$ present in ambient PM_{10} and TSP have been used; however, the use of ratios for $PM_{2.5}$ to PM_{10} or to TSP have been criticized since the ratios are different depending on region and season (Moolgavkar and Luebeck, 1996; McClellan and Miller, 1997). This contributes uncertainty to the exposure estimates for the epidemiological studies. Further, studies have shown adverse health effects to be associated with a variety of lag days (e.g., effects on the same day, or up to seven days following an air pollution episode), which leads to question why the response is not consistent in the epidemiological studies. In addition to this, a recent epidemiological study that considered multiple gaseous pollutants found much of the health effects could be attributed to the gaseous pollutants rather than particulates (Burnett *et al.*, 1999). This recent focus on other gaseous pollutants may show that increases in ambient particles are not the only cause for increased morbidity and mortality, but that there are many air pollutants contributing to adverse health effects.

It is not known at the present time whether natural particles, such as those from windblown dust, cause health effects similar to anthropogenic particles, such as those from car exhaust. Although in a recent study, Schwartz *et al.* (1999) found that coarse particles from windblown dust did not have significant effects on mortality in Spokane, WA. It is not understood which of the constituents of particulates are responsible for health effects (McClellan and Miller, 1997).

While epidemiological studies have found adverse health effects are associated with increases in ambient particulate levels, there is no known threshold for effect. The lack of an observed threshold for effects of particulate matter in epidemiological studies may be due to the nature of the population studied, since susceptible people and healthy people are studied together in epidemiological studies. Alternately, it could be due to the use of centrally located monitors that do not adequately represent personal exposures. Moolgavkar and Leubeck (1996) suggest the lack of exposure-response thresholds reported by many of the epidemiological studies may be a result of inadequate statistical analyses. The shape of the exposure-response relationship is unknown at the present time; and it has been pointed out that "the alternative of a non-linear threshold concentration response cannot be ruled out based on existing data" (McClellan and Miller, 1997). This is particularly important since there is no biological mechanism for toxicity to suggest no threshold down to zero. McClellan (1997) indicated that while different models can be used to estimate the relative risk for mortality, which assume a threshold or no threshold, until the biological mechanism of toxicity is understood, none of the models proposed can be chosen as the "correct" model.

In summary, while the results of epidemiological studies show an association between increased levels of particulate matter and other gaseous pollutants and illness or death, they do not prove causality. The establishment of a biological basis for the mechanism of action would provide evidence in support of an inference of causality. This, in association with the numerous confounding factors, and concerns regarding the statistical methods used, indicate that there is a requirement for further research into this area. Controlled human exposure studies and toxicological studies with concentrated ambient particles are necessary to identify whether the increase in particulate matter and cardiorespiratory disease are causally related, and to elucidate the biological mechanisms of toxicity.

1.3 Controlled Human Exposure Studies

In a recent workshop, Utell and Drew (1998) reported that there are not many clinical studies available for particulate matter, and most of those studies that have been conducted used acid aerosols and their salts. These studies illustrated that there were no significant effects of exposure to acidic particles (Frampton *et al.*, 1992; Anderson *et al.*, 1992). However, these studies cannot simulate what people are exposed to in ambient air since the particles in ambient air are not homogenous. Similarly, information from studies with diesel exhaust has some relevance, but while diesel exhaust particles are present in air, they are only one component of the very complex mixture of ambient particulate matter to which people are exposed. In ambient air, particles are chemically complex and heterogeneous, and are variable both temporally and spatially. The exposures of people to ambient particles are the most relevant to actual human exposure.

Several years ago, a device was developed that allowed ambient particles from outdoor air to be concentrated, without concentrating ambient gases (Sioutas *et al.*, 1995). This device (the Harvard concentrator) has made it possible to study the effects of "real-world" ambient particles for the first time, while not concentrating gaseous pollutants and therefore minimizing the exposure to ambient gases that may confound the study. Utell and Drew (1998) suggested that the initial clinical studies with ambient particulate matter should begin with healthy volunteers at ambient levels and use repeated exposures, although it is recognized that healthy, young people may not respond to low levels of particulates (Lipfert, 1997).

The only controlled human exposure studies with published data for concentrated ambient $PM_{2.5}$ (CAP) have been conducted by the US EPA in North Carolina (Ghio *et al.*, 1998; Ghio and Devlin, 1999) and the studies described in this thesis at the Gage Occupational and Environmental Health Unit, University of Toronto (Petrovic *et al.*, 1999; Urch *et al.*, 1999; Petrovic *et al.*, in press; Petrovic *et al.*, submitted). Additionally, preliminary studies are currently underway in California (Gong *et al.*, 1999).

In an initial study, Ghio *et al.* (1998) exposed eight healthy adults to concentrated ambient $PM_{2.5}$. There were no significant effects on lung function (FVC or FEV_1), no cellular inflammatory response in the respiratory tract (e.g, bronchalveolar lavage), and no cardiac abnormalities (e.g., ECG). Subjects were exposed to levels ranging from 40 to 330 $\mu g/m^3$ CAP for two hours with intermittent exercise (Ghio *et al.*, 1998). The most recent findings from this facility also did not observe a positive association with lung

function or inflammation in healthy volunteers with CAP levels of 50 to 500 $\mu g/m^3$, using a similar protocol (Ghio and Devlin, 1999).

Therefore, it can be seen that there is a paucity of data regarding controlled exposures to ambient particles. The available controlled human exposure studies cannot be used to support the results of the epidemiological studies that show adverse cardiorespiratory effects at ambient levels of particulate matter (Utell and Frampton, 1995; McClellan and Miller, 1997). Further research is required in this area. Moreover, knowledge regarding potential additive or synergistic effects of exposure to $PM_{2.5}$ with gaseous pollutants is currently lacking. In particular, ground level ozone is a common pollutant that triggers smog advisories in Toronto, and has been shown in clinical exposure studies to have adverse effects on lung function as well as respiratory symptoms at levels from 80 to over 200 ppb (Koenig, 1999). Some recent studies with ozone, similar to those conducted for this thesis, are briefly summarized below.

Controlled human studies with ozone have shown effects on pulmonary function and respiratory symptoms. Not all people respond to O_3, and subgroups of "ozone responders" have been identified that are more susceptible to the effects of O_3; however, it is not known why some people are more sensitive than others (McDonnell, 1996). Controlled human exposure has been found to decrease lung function and increase respiratory symptoms following exposure to O_3 at 0.22 ppm for four hours with exercise (Frampton *et al.*, 1997). Greater effects were seen in non-smokers compared with smokers. Another study found a four hour exposure to 0.2 ppm O_3 resulted in increased respiratory symptoms that were not associated with significant decreases in FEV_1 (Aris *et*

al., 1995) and suggested that people could experience increased respiratory symptoms without significant lung function decline. A later study showed a decrease in FEV_1 and FVC but no airway reactivity in 45 middle aged non smoking adults at 0.4 ppm O_3 for two hours with exercise (Gong *et al.*, 1998a).

Recently, the cardiac effects of exposure to 0.3 ppm O_3 for three hours with intermittent exercise was studied in six healthy men and ten men with hypertension (Gong *et al.*, 1998b). No significant effects were seen on the ECG. There was increased heart rate seen in the subjects exposed to O_3 compared with filtered air. The authors suggested this might adversely affect people with cardiorespiratory disease.

Ozone is an oxidant pollutant that can cause damage to the bronchial epithelium and an influx of neutrophils into the airways during an acute inflammatory response (Krishna *et al.*, 1998). Neutrophils and fibrinogen were elevated in BAL fluid of normal nonsmoking adults exposed to 0.35 ppm O_3 for two hours (Weinmann *et al.*, 1995). The increase in BAL fibrinogen levels was associated with small airway dysfunction, measured as a reduction in FEF_{25-75}. A recent study found an increase in sputum neutrophils post-exposure to 0.125 and 0.25 ppm O_3 (Holtz *et al.*, 1999), suggesting an acute inflammatory response in the lungs following O_3 exposure at these levels. McBride *et al.* (1994) observed that asthmatics were more sensitive to O_3 than non-asthmatics, with an increase of neutrophils in nasal lavage fluid. Other studies have also found an immediate increase in nasal lavage neutrophils following O_3 exposure (Graham *et al.*, 1988; Koren *et al.*, 1990), but with a lot of intraindividual variability on different days (Koren *et al.*, 1990). A recent study did not find an increase in nasal lavage neutrophils in healthy

volunteers exposed to 0.2 ppm O_3 for 2 hours with intermittent exercise (Mudway *et al.*, 1999). Similarly, another study did not find increases in neutrophils in BAL fluid after exposure to 0.2 ppm O_3 for two hours with intermittent exercise, although there was a decrease in BAL macrophages (Blomberg et al., 1999). In this study, there were decreases in lung function, evidenced by FVC and FEV_1 measurements.

Therefore, numerous studies have shown that exposure to elevated O_3 levels can cause adverse human health effects. Further study is required to examine the effects of $PM_{2.5}$ on healthy and more susceptible populations in order to identify the levels of $PM_{2.5}$ that cause adverse effects in controlled exposure studies. Additionally, the human health effects of exposure to combinations of pollutants is another important area of study that is in its infancy.

1.4 Animal Toxicology Studies

Few toxicological studies have been conducted with animals exposed to concentrated ambient $PM_{2.5}$ (CAP). Most of the studies in animals have been conducted with artificial particles, re-suspended flyash or urban dust (Lipfert, 1997; McClellan, 1997). These studies are important to the investigation of the toxicity of particulate matter, but there are many differences with artificial or flyash particles compared with ambient particles. The chemical composition is an important factor in the consideration of toxicity of ambient particles, and can not be practically replicated. The exposure of animals to CAP in toxicology studies is important because these particles are similar to those found in ambient air. Therefore, for the purpose of this summary, greater weight will be given to studies with CAP.

Cardiorespiratory effects in animal studies with particles include increased morbidity and mortality; however, it is noted that the level of exposure of animals to particles in these studies is much higher than found in ambient air. Mean levels of $PM_{2.5}$ in Toronto are approximately 20 $\mu g/m^3$, while levels in these studies are an order of magnitude higher. The levels of particles used in animal toxicology studies are generally above 200 $\mu g/m^3$, and can range up to mg/m^3 levels, as described below. There are problems in extrapolating results from exposure levels orders of magnitude higher than ambient to effects at ambient levels, since different biological mechanisms of action may operate when the respiratory system is overloaded with particles.

1.4.1 Concentrated Ambient Particles (CAP)

Godleski *et al.* (1996) exposed rats to CAP in Boston air, using a method to concentrate ambient particles, described by Sioutas *et al.* (1995). Rats were separated into three groups: i) normal; ii) SO_2-induced chronic bronchitis; and iii) monocrotaline-induced inflammation (i.e., a chemical that causes lung injury that can lead to pulmonary hypertension and right heart enlargement, creating an animal model for COPD in humans). Each group was exposed to mean concentrations of 228, 245 or 288 $\mu g/m^3$ CAP for six hours per day, for three days. Increased mortality was found in compromised rats with bronchitis (37%), and inflammation (19%) compared with healthy rats (0%). All rats with bronchitis were reported to have increased bronchoconstriction, compared with normal rats that had minimal bronchoconstriction and no inflammation. The results from this study indicate that animals with pre-existing cardiorespiratory disease are more susceptible to CAP toxicity than healthy animals.

In a later study, decrements in lung function were found in healthy rats and rats with bronchitis exposed to CAP from Boston air at exposure concentrations of 206, 733 and 607 $\mu g/m^3$ for six hours/day for three days, respectively (Clarke *et al.*, 1998a). Pulmonary inflammation, as assessed with cells from bronchoalveolar lavage fluid (BAL), showed an increase in neutrophils and lymphocytes in both normal and bronchitic rats and an increase in BAL protein in bronchitic rats. Again in this study, all changes appeared greater in rats with bronchitis (Clarke *et al.*, 1998b).

Dogs exposed to CAP at a mean level of 193.9 $\mu g/m^3$ for six hours/day for three days had elevated concentrations of neutrophils in BAL post-exposure when compared with baseline values (Godleski *et al.*, 1997). Additionally, one of the four dogs had altered electrocardiograms (ECG) during exposure to the concentrated particles. The significance of this finding is uncertain due to the small number of subjects in the study. The authors concluded that ambient particles may cause adverse cardiorespiratory effects. It is noted that the data from these studies are not available to the author and the results have not yet been published in a peer reviewed article.

Lovett *et al.* (1998) found that HRV was affected in dogs exposed to CAP at mean levels of 190 $\mu g/m^3$ for six hours per day for 28 days. The authors suggested there may be increased vagal activity of the autonomic nervous system following exposure to CAP based on a higher ratio of high frequency power (HF) to total power (TP) (refer to Section 4.5 for a fuller description of these terms) when compared with filtered air exposures. The authors indicated that altered breathing patterns during CAP exposures may have resulted in the altered HF/TP ratio.

1.4.2 Other Particulate Matter and Gaseous Pollutants

Exposure of rats to ambient PM_{10} (collected in the U.K.) via intratracheal instillation resulted in proinflammatory effects such as increased neutrophils, increased epithelial permeability and increased total protein in BAL fluid six hours following exposure (Li *et al.*, 1997). In another study, increases in BAL neutrophils were found following instillation of Washington, D.C. ambient particulate matter in rats. There were also increases in protein and eosinophils (Dreher *et al.*, 1996).

Effects of exposure to more than one pollutant are also of interest since there are many chemicals present in air. Bouthellier *et al.* (1998) has studied the effects of exposure to resuspended urban dust and/or ozone in rats. Inhalation of 40 mg/m^3 urban dust (mean median diameter of 4 to 5 μm) for 4 hours did not result in acute lung injury of rats. Rats exposed to ozone (0.8 ppm) had an inflammatory response, with increased BAL neutrophils and protein. Co-exposure of rats to urban dust with ozone resulted in potentiation of the ozone effects by particles. Thus it is important to study the additive or synergistic effects of ambient particles with gaseous pollutants.

Many of the toxicological animal studies have been conducted with re-suspended flyash, which is released from combustion (e.g., of fuel oil or coal). Flyash is present in ambient air from electricity-generating stations, but it is mixed with particles from other sources. Re-suspended flyash has different composition than ambient air particulates, particularly elevated leachable metals and sulfates (Prichard *et al.*, 1996).

A number of studies have shown pulmonary effects following inhalation of flyash. One study exposed healthy rats and compromised rats with pulmonary injury induced by monocrotaline injection to flyash via inhalation. Exposure for six hours/day for three days at mean particle levels of 580 $\mu g/m^3$ (2.06 μm median diameter) resulted in increased mortality (42%) in compromised rats but not healthy rats (Killingsworth *et al.*, 1997). Pulmonary inflammation in compromised rats was increased with flyash exposure, as evidenced by increases in neutrophils. Increases in neutrophils, macrophages and eosinophils were also found in the lungs of rats exposed to 0.04, 0.2, or 1 mg oil flyash (Gilmour *et al.*, 1998). In another study, rats exposed to flyash particles also had increased pulmonary inflammation, with an influx of neutrophils into the alveoli which reached its peak at 24-hours post-exposure (Jascot *et al.*, 1995). There was a slight increase in mortality in rats with pre-existing inflammation exposed to flyash via intratracheal instillation, but not in rats with pre-existing fibrosis (Kodavanti *et al.*, 1996).

Several studies with flyash have shown cardiac effects in animals. In rats exposed to 0, 0.25, 1, or 2.5 mg/mL flyash via intratracheal instillation, cardiac effects in healthy and monocrotaline-treated pulmonary hypertensive rats included increased arrhythmias and skipped heart beats in a dose-dependent manner (Campen *et al.*, 1996). In another study using the same exposure levels, increased arrhythmias were more severe in monocrotaline-treated rats compared with healthy animals, and there was increased mortality in monocrotaline-treated rats (Watkinson *et al.*, 1998a). Similarly, monocrotaline-treated rats exposed to 15 mg/m^3 flyash (six hours/day for three days) via inhalation had arrhythmias and ST segment depression potentiated by particulate exposure (Watkinson *et al.*, 1998b), but no mortality. No ECG abnormalities were

observed in healthy rats, and the authors concluded the flyash exposure was moderately cardiotoxic in compromised rats. In dogs, flyash exposure (1.68 and 3.04 mg/m^3) resulted in increased amplitude of T wave alternans and increased ST segment elevation (Nearing *et al.*, 1996). This result was reported to be consistent with cardiac inflammation; however, there was no histological evidence of cardiac inflammation.

In addition to ECG evidence of cardiotoxicity, cardiovascular illness is also associated with increased plasma fibrinogen levels. Plasma fibrinogen levels and leukocyte counts were increased 24-hours post-exposure in rats exposed to 8.3 mg/kg flyash by intratracheal instillation (Gardner and Costa, 1998). The increase may be due to increased alveolar inflammation following exposure to particles, resulting in increased blood coagulation. There were no data found in the literature reviewed for this thesis regarding plasma fibrinogen levels following exposures to the air pollutant, ozone.

Rats and monkeys exposed to high concentrations of particles from diesel exhaust and coal dust in chronic studies were reported to have differential uptake and retention of particles (Nikula *et al.*, 1997). Interspecies differences included increased retention of particles in the lumen of alveolar ducts and alveoli in rats and significant inflammatory, fibrotic, and hyperplastic response to particles. This compared with results in monkeys, which had significantly more particles in the interstitium than rats, but no other significant effects of exposure. Therefore, interspecies differences must be taken into account when interpreting data from animal toxicology studies.

The biological mechanisms for the cardiorespiratory effects of particulate matter are not understood presently (Environment Canada, 1997). It is also not known whether the

mass, size or composition of the particles is most important for toxicity (Reichhardt, 1996; Brook *et al.*, 1997). Although there has been a lot of research recently to identify potential mechanisms of toxicity of ambient particles, no mechanisms have yet been elucidated and nor have any been excluded. It is apparent from epidemiology studies that the mechanisms involve effects on the respiratory and cardiovascular systems. From epidemiological studies, susceptible populations likely include asthmatics, COPD patients and people with cardiovascular disease (Utell and Drew, 1998). Further research into the health effects of $PM_{2.5}$ is required.

Based on the effects observed in epidemiological studies, tests were identified that can identify potential cardiorespiratory effects in controlled human exposure studies. These include assessment of pulmonary function, respiratory tract lining fluid, blood analyses, and HRV and ECG measurements. These will be described further in Section 4.

2.0 OBJECTIVES

The overall objective for this thesis was to conduct preliminary controlled human exposure safety studies with concentrated ambient $PM_{2.5}$ (CAP) to identify potential cardiorespiratory effects following acute inhalation exposure. The first study was conducted with exposure of young, healthy individuals with CAP alone, and the second study was to study the effects of CAP and/or ozone exposure in young, asthmatic adults. The primary objective was to examine potential health effects in young, healthy or mildly asthmatic adults to CAP at concentrations at or above those found in Toronto ambient air in these pilot safety studies. The intent of the experiments was to study of the effects of exposure to concentrated ambient particles with the same physical/chemical characteristics of actual outdoor exposures. An additional objective was to examine whether there are synergistic or additive effects of combined exposure to ozone and CAP. These pilot studies were designed to identify CAP concentrations for use in future studies with a greater number of individuals.

3.0 HYPOTHESES

Given that epidemiological studies have found increased cardiorespiratory effects following exposure to elevated ambient particles, it is hypothesized that acute exposure to CAP will result in effects in controlled human exposure studies. Cardiorespiratory effects may include:

- An inflammatory response in both healthy and mildly asthmatic adults, as reflected by an increase in inflammatory markers (e.g., neutrophils) in respiratory fluids, with a stronger response expected in asthmatics.

- Combined exposure to CAP and ozone is expected to produce a stronger respiratory response than CAP or ozone alone.

- An increase in blood clotting activity (e.g., fibrinogen) is expected to follow exposure to CAP, as an indicator of inflammation.

- While cardiac effects have been seen in susceptible populations, it is not expected that there would be clinically significant cardiac effects evident on an ECG in young, healthy or mildly asthmatic individuals.

- Decreased HRV may occur in people exposed to high levels of CAP, since decreased HRV is associated with increased mortality in susceptible populations.

- Not all subjects are expected to respond, based on results from epidemiological studies that have found effects in some individuals but not others.

4.0 METHODOLOGY

Two separate studies are presented in this thesis. The first study (Study No. 1) was conducted in 1998 with young, healthy individuals. The second study (Study No. 2) involved the use of atopic, asthmatic subjects in 1998 and 1999. The protocol for the second study was similar to that of the first, although it was altered based on results from the first study described in Section 5. Results from Study No. 2 are presented in Section 6, and a discussion of the results from both studies is provided in Section 7.

In order to determine health effects of ambient particles, we identified important tests for human clinical studies. These were similar to those identified by Utell and Drew (1998) at a workshop of experts in the field and included: i) pulmonary function (spirometry, airway resistance, indices of gas exchange); ii) respiratory tract lining fluid (nasal lavage, sputum induction); iii) blood analyses; iv) HRV and ECG measurements; and v) physical/chemical analyses of particulate matter used in studies. The tests used for the purpose of this thesis are described further in this section.

4.1 Subject Selection

Although it is recognized that most of the effects in epidemiological studies are seen in elderly or cardiorespiratory-compromised individuals, pilot tests with CAP are necessary with young, healthy individuals to determine "safe" exposure levels. Ethically, only minor, reversible effects are acceptable in subject populations and thus young healthy or mildly asthmatic adults, who are most likely to not have serious consequences following exposure in pilot studies were used in the initial pilot studies.

Four young, healthy non-smoking males and female volunteers, between the ages of 18 and 40 years were recruited for the first study, and four asthmatic atopic volunteers of the same age were selected for the second study, all from the downtown Toronto area. In order to minimize the potential for confounding inflammation from respiratory infection, it was confirmed that subjects had not had any acute respiratory illness for at least three weeks prior to study enrollment or exposures.

A baseline test was conducted to confirm the subjects were healthy and did or did not have asthma for each study. It was established that the subjects did not have cardiac or respiratory conditions and were able to participate in the study (see Section 4.2). Baseline subject characteristics are shown in Table 4-1. In Study No. 1, two male and two female subjects without asthma were recruited. All subjects for this study were 20 years old, with a mean height of 171 cm and weight of 66 kg. The fibrinogen and Factor VII values were in the normal range for the subjects. In Study No. 2, all of the asthmatic subjects recruited were female, with a mean height and weight of 167 cm and 59 kg, respectively. The fibrinogen and Factor VII values were in the normal range for the subjects. The severity of the asthma in each subject was variable.

4.2 Baseline Testing

As mentioned above, the subjects were tested in a four-hour baseline visit prior to the commencement of the study. A physical examination was conducted by a physician and a medical history questionnaire was completed at this visit. Subjects were excluded if the physician identified significant abnormalities. A battery of tests was performed to

confirm that pulmonary function was within normal limits (see below) and that each subject met the inclusion criteria and became familiar with the tests used in the studies.

For Study No. 1 with healthy individuals, baseline tests included: i) methacholine challenge to confirm the subjects were not asthmatic (PC_{20}>16.0 mg/ml); ii) lung function tests including flow-volume curve (>75% of predicted normal values), lung volumes (>75% of predicted), airway resistance (<3cm H_2O/L/sec), and lung diffusing capacity (>75% of predicted); iii) resting ECG to confirm no cardiac abnormalities as evaluated by a cardiologist; iv) exercise test to confirm ability to ride a bicycle for 30 minutes, to determine workload for 65% predicted maximum heart rate, and to confirm no cardiac abnormalities; v) blood tests to confirm no abnormal coagulant activity (i.e., fibrinogen and factor VII); vi) induced sputum to obtain cells from the lower airways for total and differential cell counts and protein concentration; vii) nasal lavage to obtain cells from the nasal cavity for total and differential cell counts and protein concentration; and viii) a nasal acoustic rhinometry test.

The same baseline tests were conducted for Study No. 2, with similar inclusion criteria to Study No. 1, but with a few exceptions since subjects were selected to be atopic asthmatics with eosinophilia. The methacholine challenge was confirmed to be PC_{20} < 8 mg/ml, which is indicative of asthma. Eosinophilia was identified from induced sputum samples (i.e., more than 3% eosinophils). Based on results from Study No. 1 (see Section 5), plasma clotting Factor VII antigen was not analyzed.

4.3 Human Exposure Facility (HEF)

We have developed a concentrated ambient particle HEF similar to that used in recent animal toxicological studies with CAP (Godleski *et al.*, 1996, 1997) and human studies with CAP (Ghio *et al.*, 1998; Ghio and Devlin, 1999). The HEF, shown in Figure 4-1, concentrates particles using a sequential virtual impactor system that is essentially identical to the design described by Sioutas *et al.* (1995) except for inclusion of only two stages. Two stages were sufficient to obtain the CAP concentrations desired for these studies. This facility is equipped with a 2.5 μm size-selective inlet to obtain only particles smaller than 2.5 μm in aerodynamic diameter. The lower cut-point of the concentrator is 0.1 μm, since the ultrafine particles are drawn off with the gases and not concentrated. In this manner, outdoor concentrations of fine ambient particles in the size range of 0.1-2.5 μm (aerodynamic diameter) from downtown Toronto were concentrated to simulate levels similar to or higher than typical smoggy day concentrations in Toronto. Smoggy day concentrations of $PM_{2.5}$ in Toronto are approximately 60 $\mu g/m^3$, and the exposure levels in the studies were up to 130 $\mu g/m^3$. Evaluation of the sequential virtual impactor technology by Sioutas *et al.* (1995) has indicated that the chemical properties of the particles are not substantially modified. The design of the HEF is not part of the mandate for this thesis and will not be discussed in detail in this document. The reader is referred to Sioutas *et al.* (1995) and the Masters thesis of Mr. Jason Datema, University of Toronto (1999) for further details.

A relatively stable CAP concentration was maintained over the two hour exposure by a controlled addition of HEPA/charcoal filtered indoor air to the ambient air inlet to dilute

Table 4-1: Subject Baseline Characteristics

Subject Number	Sex	Age (yrs)	Height (cm)	Weight (kg)	FEV_1 (% predicted)	Methacholine PC_{20} (mg/ml)	Fibrinogen (mg/dl)[1]	Factor VII Antigen (%)
Study No. 1								
PS2	F	20	173	75	110	>16	340	88.4
PS3	F	20	160	54	108	>16	337	98.2
PS5	M	20	177	66	108	>16	220	99.6
PS7	M	20	173	70	105	>16	224	92.5
Mean	-	**20**	**171**	**66**	**108**	**>16**	**280**	**94.7**
SE	-	0	4	4	1	0	34	2.6
Study No. 2								
PO2	F	20	165	63	111	3.2	183	NA
PO5	F	22	163	56	116	0.2	213	NA
PO6	F	29	180	64	87	0.7	200	NA
PO7	F	25	160	52	104	2.0	172	NA
Mean	-	**24**	**167**	**59**	**105**	**1.5**	**192**	-
SE	-	2	4	3	6	0.7	9	-

1) Note that methodology for fibrinogen varied between Study No. 1 and Study No. 2 (see Section 4.7)

the particle mixture during transient increases in the inlet ambient $PM_{2.5}$ (see Figure 4-1). Particulate mass concentrations were measured continuously during exposure using two real-time aerosol monitors (each monitor is a TSI DustTrak, model 8520). One was located on the window ledge in the ambient air by the concentrator inlet, and the other in the CAP air stream just prior to delivery to the subject (Port 2). These measurements were necessary to regulate the particle levels during exposure in order to achieve the target particle exposures and for subject safety. The DustTrak, which determines particle concentrations based upon light scattering, was calibrated on site through comparison with gravimetric measurements. This helped to ensure that the real-time particle measurements and the subsequent settings of the CAP levels were as close to the actual particle exposures as possible. However, given that light-scattering measurement is at best an indicator of particle mass concentration, the port 4 gravimetric measurements were taken as the actual subject exposure.

During human CAP exposures, in addition to the aerosol monitor, particles were collected on 47 mm (2 μm pore size), Teflon (PTFE) filters from the ambient air (using a $PM_{2.5}$ size selective inlet) and in the CAP air stream (Port 4). Filters were pre- and post-weighed after conditioning for 48 hours at approximately 30% RH and 22°C (Perkin Elmer, model AD-6 electrobalance). Exposure to gaseous criteria pollutants (Port 3), and the temperature, relative humidity and CO_2 (Port 1) were measured in the CAP airstream.

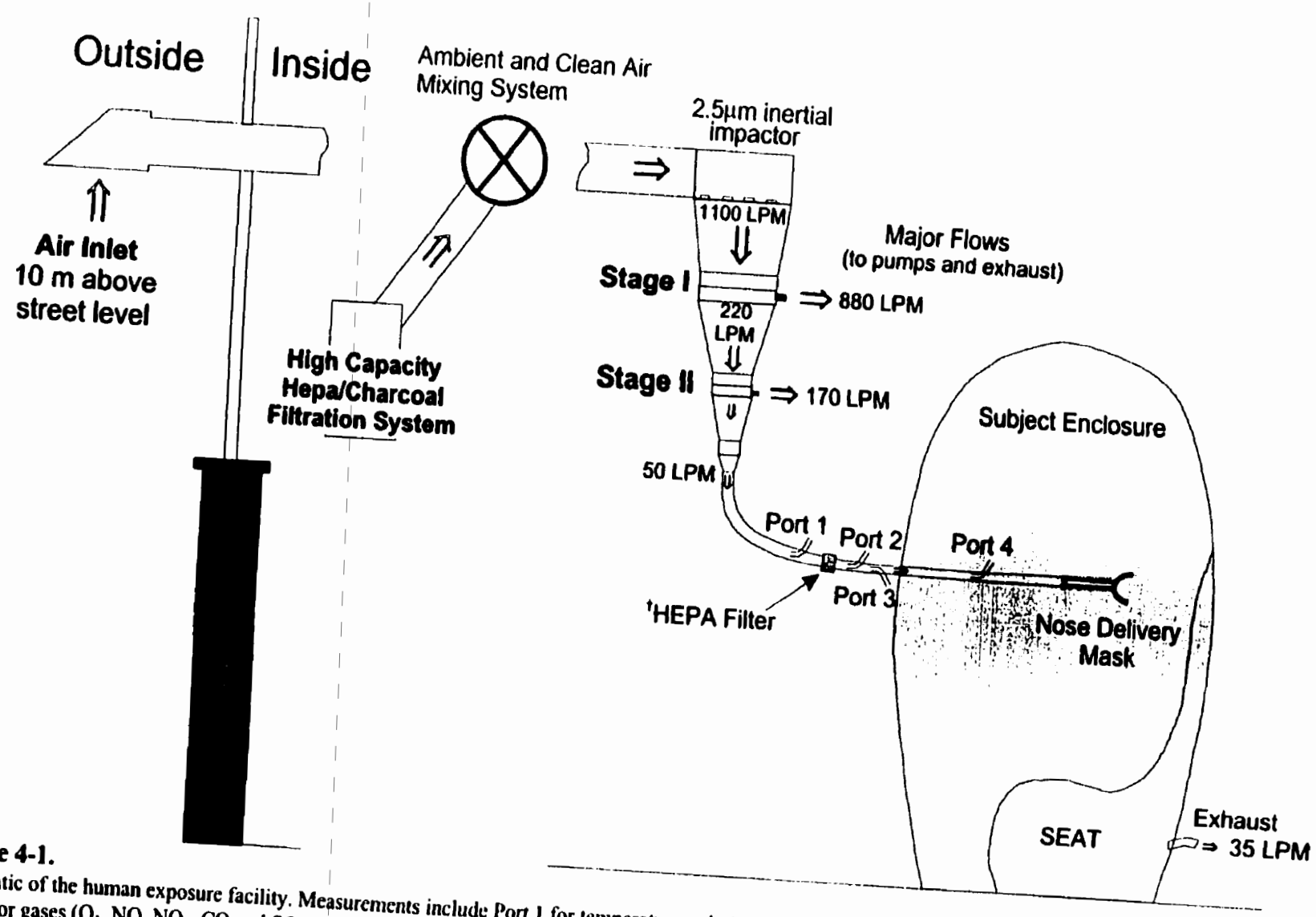

Figure 4-1.
Schematic of the human exposure facility. Measurements include Port 1 for temperature, relative humidity (%) and CO_2; Port 2 for real-time aerosol monitor; Port 3 for gases (O_3, NO, NO_2, CO and SO_2); and Port 4 for filter sample at 15 L/min. A †HEPA filter cartridge was inserted inline in the CAP airstream for filtered air exposures.

The interior of the HEF, where each subject sat during exposures, was a modified oval-shaped plethysmograph cabinet (OHIO Medical Products, model 310) and had an interior volume of 800L. The air was exhausted from the HEF at a flow rate of 35 L/min. There was a slight negative pressure that varied from 4.5 to 5.5 inches of water inside the enclosure.

The CAP air stream was delivered directly to the subjects using an "oxygen-type" mask (Baxter Airlife, aerosol mask) that covered the nose and mouth but was open at the bottom. The mask was connected to the CAP air stream via a flexible tube (VacuMed, Clean-Bor 1011-10) which had a smooth interior wall. Calibration tests confirmed that mass concentration measurements obtained from the aerosol monitor sampling from the CAP airstream at Port 2 (Figure 4-1) were the same as that inside the mask. Subjects were asked to breathe through the nose during the exposures.

4.4 Study Design

4.4.1 Study No. 1

In Study No. 1, each of the subjects was exposed on separate days to filtered air (FA) and target concentrations of 20, 40, and 60 $\mu g/m^3$ CAP for 2 hours. For Study No. 1, these target exposure levels are referred to as FA, Low CAP, Mid CAP and High CAP. These CAP levels were chosen to represent mean Toronto ambient air concentrations of $PM_{2.5}$ (20 $\mu g/m^3$) and smoggy levels of $PM_{2.5}$ (60 $\mu g/m^3$) (Brook *et al.*, 1997). An exposure in between of 40 $\mu g/m^3$ was also used. This was chosen since increments of 10 $\mu g/m^3$ have been reported to have increased health effects in epidemiological studies. These exposures should also provide information for dose-response effects based on mass

concentration covering a range of typical ambient levels of $PM_{2.5}$ in Toronto. During FA exposure a HEPA filter (Wilson, respirator cartridge) was inserted inline in the CAP airstream before delivery to the subject (see Figure 4-1). When the HEPA filter was used, the negative pressure inside the enclosure ranged from 5.5 to 6.5 inches of water.

Exposures were at rest, and for safety reasons, the order of exposures was non-randomized. Subjects were first exposed to FA, followed by the low CAP, then mid CAP, and lastly the high CAP concentration. The subjects as well as the technologists (with the exception of the technologist conducting the exposures) were blind as to the exposure condition. A washout period of two weeks was allowed between exposures to ensure no residual effects of exposures that may confound the study, with generally no more than four weeks between exposures. Additionally, at the time of each exposure, it was confirmed that the subjects had not had acute respiratory illness at least three weeks prior to each exposure in order to minimize the potential for confounding inflammation from respiratory infection.

On each exposure day for Study No. 1, tests prior to exposure included: pulmonary function, nasal lavage, nasal acoustic rhinometry, blood collection for plasma fibrinogen and clotting factor VII antigen, and a resting ECG. During exposures, the subjects were continuously monitored through three plexiglass windows in the HEF, lung function was assessed with a portable spirometer, and cardiac effects were monitored continuously with a 12-lead ECG. Following exposure, tests included: lung function, symptom report, nasal acoustic rhinometry, blood collection post-exposure, a 30-minute exercise test, nasal lavage, sputum induction, a resting ECG and blood collection also post-exercise. A

Holter monitor was used for HRV analysis for the entire exposure day. Approximately 24-hours following exposure, the following tests were conducted: lung function, symptom report, sputum induction, nasal lavage, blood collection, and a resting ECG.

4.4.2 Study No. 2

In Study No. 2, exposure concentrations were selected based on results from Study No. 1 (see Section 5). The results from Study No. 1 indicated that 60 $\mu g/m^3$ should be a "safe" exposure level for the asthmatic subjects. Asthmatic subjects were exposed to target concentrations of filtered air (FA), the High CAP level of 60 $\mu g/m^3$ (CAP), the regulatory limit of 80 ppb ozone (O_3) or a combination of CAP+O_3 (*e.g.*, 60 $\mu g/m^3$ CAP + 80 ppb O_3). These exposures were in randomized order, except for the combined exposure following the individual CAP and O_3 exposures. This was carried out for safety reasons since this was the first controlled human exposure study conducted with a combination of CAP and O_3.

The study design was similar to Study No. 1. The following modifications were made in Study No. 2: i) sputum induction was performed on the day prior to exposure to obtain pre-exposure values and minimize intra-individual variability; ii) a methacholine challenge was performed on the day pre-exposure and 24 hours post-exposure to assess airway hyperreactivity in asthmatic subjects; iii) plasma clotting Factor VII antigen was not analyzed based on results from Study No. 1 (see Section 5); and iv) a resting ECG was not taken 24-hours post-exposure, also based on results from Study No. 1 (see Section 5).

One of the subjects (PO5) in Study No. 2 did not complete all exposures. This subject developed frequent asthma exacerbations due to several colds and allergies to indoor mold in the home towards the end of the study period. For this subject, we obtained only two exposures (CAP and O_3). Due to limitations in the funding deadline, there was insufficient time to enroll a new subject into the study and complete all exposures. A consensus was reached with the funding agency that we add a Max CAP exposure of approximately 120 $\mu g/m^3$ for two of the completed subjects (PO2 and PO6). This provided an assessment of the effects of exposure to higher levels of CAP in subjects for whom we had data at lower CAP levels. One of the remaining subjects (PO7) was not able to participate in the last exposure due to seasonal allergies. We did not expose any atopic subjects to CAP during their allergen season to minimize confounding and to prevent potential health complications. The revised exposure protocol for Study No. 2 is summarized below:

Subject	FA	CAP	O_3	CAP+O_3	Max CAP
PO2	✓	✓	✓	✓	✓
PO5		✓	✓		
PO6	✓	✓	✓	✓	✓
PO7	✓	✓	✓	✓	

4.5 ECG/Heart Rate Variability

The electrocardiogram (ECG) is used to monitor cardiac function. If there is acute cardiotoxicity (e.g., adverse effects on the heart) associated with exposure to ambient

particles, then there may be changes in ECG waveforms. Changes in the ECG, indicative of myocardial ischemia (e.g., decreased blood flow), have been seen in animals exposed to high levels of particles, and include ST segment depression or elevation and T wave alternans (as discussed in Section 1.4). It is also of note that occupational exposure to dusts has been associated with increased ischemic heart disease (Sjogren, 1997). Since cardiovascular effects have been shown in epidemiological studies (see Section 1.2) and in animal toxicology studies, it is important to monitor ECGs during human clinical studies.

Heart rate variability (HRV), a quantitative marker of autonomic nervous system activity, is measured using an ECG signal obtained from a Holter tape recorder (Winchell and Hoyt, 1997). Physiologic regulation of the heart is complex and involves autonomic input to the sinoatrial node. Sympathetic and parasympathetic activity from the autonomic nervous system controls the heart (Guyton, 1991). HRV is a measure of the interval between consecutive heartbeats, and is generally measured over a 24-hour period (Cardiac Task Force, 1996), although time periods from 5 minutes to 24 hours are also used. It is important to compare data from the same time intervals since the total variance of the HRV increases with the length of recording. The optimal length of recording for high frequency (HF) and low frequency (LF) is 5 minutes and for SDNN is 24 hours (Kautzner and Hnatkova, 1995). The HRV measurements must be obtained from ECG segments without arrythmias or ectopic beats (Winchell and Hoyt, 1997), since only normal-to-normal beats are reflective of autonomic input to the sinoatrial node.

HRV can be assessed with frequency domain measurements that include LF, HF and LF/HF ratio. LF is a mix of sympathetic and parasympathetic activity (Winchell and Hoyt, 1997). The LF component is a marker of sympathetic modulation, where an increase in sympathetic activity results in an increase in LF, such as with exercise (Cerutti *et al.*, 1995). Increased HF results from increased vagal activity, such as controlled respiration. The LF/HF ratio is an indicator of the sympathovagal balance. The balance of sympathetic and vagal activity is important to determine which is dominant. HRV can also be measured by time domain methods, using the normal-to-normal (NN) intervals between adjacent QRS complexes. The SDNN is the standard deviation of the NN intervals. The square of SDNN is reflective of the total power of spectral analysis that reflects all the components responsible for variability in a 24-hour recording, although this mathematical association is not true for all time intervals (Kleiger *et al.*, 1995). The rMSDD is the square root of the mean squared differences of successive NN intervals, and is an estimate of the short-term variation, or the high frequency variation in heart rate (e.g., vagal activity). The pNN50 is also a measure of vagal activity, and provides information on the proportion of cycles where the difference between adjacent cycles (e.g., the number of beat-to-beat NN interval differences) is more than 50 milliseconds (ms). These time domain and frequency domain measures are summarized in Table 4-2.

HRV is important for cardiovascular effects because there is a significant association between autonomic nervous system activity and cardiovascular mortality. Decreased HRV is a strong independent predictor of mortality (Tsuji *et al.*, 1994), although the

mechanism for this is not understood. Decreased HRV is observed as low NN interval variability, such as with SDNN.

Table 4-2: Summary of the Physiological Relevance of Some HRV Measures

Parameter	Units	Physiological Relevance
Frequency Domain		
LF	ms^2	mix of vagal and sympathetic activity
HF	ms^2	vagal activity
LF/HF ratio	unitless	sympathovagal balance
Time Domain		
SDNN	ms	overall HRV
RMSDD	ms	vagal activity
pNN50	%	vagal activity

For the purpose of this study, the subjects were connected to a 12-lead ECG monitor (Marquette, Case-16) using a modified Mason-Likar exercise electrode placement to allow for subject movement and exercise throughout the day. A resting 12-lead ECG printout was obtained at the beginning and end of each exposure day for Study No. 1 and Study No. 2. Additionally, a resting ECG was obtained 24-hours post-exposure for Study No. 1. Based on results in Study No. 1, a resting ECG was not obtained 24-hours post-exposure for Study No.2. The ECG was monitored continuously for safety reasons during exposure, the 30-minute exercise period, and recovery following exercise. Printouts of the 12-lead ECG were obtained every 10 minutes during exposure and every 5 minutes during exercise. The ECG printouts were reviewed by a cardiologist (Dr. E. Downar at

Toronto General Hospital) for waveforms indicative of silent myocardial ischemia, including ST segment depression and T-wave alternans. The purpose of the 12-lead ECG was to provide a real-time display/printout of the ECG for safety reasons during exposure and exercise, and was not intended for heart rate variability (HRV) data analysis. The HRV data were obtained from the Holter recorder, described below.

Subjects were connected to a Holter recorder (7-lead) to monitor HRV over each exposure day (approximately seven hours). The leads were placed in the exact same location for each exposure day. The Holter tapes were analyzed for HRV during the two-hour exposure and 30-minute exercise periods using a Mars 8000 Work Station (Marquette Electronics). The QRS complexes were labeled, and a trained technician edited/removed ectopic heartbeats so that only normal RR intervals were isolated from the tapes prior to obtaining the HRV data, as recommended by Kleiger *et al.* (1995). It is important to remove ectopic heartbeats since HRV is analyzed from normal heartbeats generated from the sinoatrial node (e.g., input received from the autonomic nervous system). Ectopic heartbeats were assessed for clinical significance on the 12-lead ECG.

For Study No. 1, the Holter tapes were analyzed at Harvard. Minute to minute variations were provided for heart rate (beats/min), SDNN (beats/min), LF $(\text{beats/min})^2$, HF $(\text{beats/min})^2$ and LF/HF ratio (unitless). The frequency range for LF and HF was 0.04-0.15 Hz and 0.15-0.4 Hz, respectively. The minute-to-minute data were averaged using an Excel spreadsheet for the 120-minute exposure period and the 30-minute exercise period. It is noted that the data were provided in units that are not the standard units (as provided in Table 4-2).

The tapes from Study No. 2 were analyzed at St. Michael's Hospital, Toronto. Again, both time domain (average heart rate, SDNN, rMSDD, pNN50) and frequency domain (LF, HF, LF/HF ratio) parameters were obtained from the tapes. Reports were obtained for these parameters using the MARS 8000 software program, and the units for the parameters were selected based on the Cardiac Task Force (1996): heart rate (beats/min), SDNN (millisecond, ms), rMSDD (ms), pNN50 (%), LF (ms^2), HF (ms^2) and LF/HF ratio (unitless). Similar to the first study, data were obtained for the 120-minute exposure and 30-minute exercise period.

The data from the HRV analyses for each study were obtained using different statistical methods. The method used by Harvard is different from that used in the MARS 8000 software program. Additionally, the data from Harvard was provided as cardiac beats, rather than in milliseconds, which are both acceptable (Cerutti *et al.*, 1995), but different. Data using different statistical methods cannot be directly compared unless they have been independently verified by another institution (Cardiac Task Force, 1996). The actual statistical programs used for these calculations is not part of the research mandate for this thesis, and therefore will not be discussed in this thesis. However, since the statistical methods differ, the data from Study No. 1 will not be directly compared with that from Study No. 2.

4.6 Exercise Test

Subjects rode a stationary Monark bicycle ergometer following each exposure to identify whether there are cardiac effects following exposure to pollutants (e.g., to bring on cardiac effects). The workload was adjusted to maintain a target heart rate at 65%

maximum heart rate for 30 minutes. For a 20-year old subject, this is 130 bpm (calculated as (220-20[age]) x 0.65). Blood pressure was monitored and a 12-lead ECG printout was obtained every five minutes. The exercise was followed by a recovery period to allow the subject heart rate to return to baseline. The recovery period lasted approximately 5 to 15 minutes, depending on the physical activity level of each subject since trained athletes return to baseline more quickly than untrained individuals.

4.7 Blood Coagulation Factors

Inflammation in tissue, including lung tissue, can result in increased blood coagulation (Guyton, 1991). Seaton *et al.* (1995) suggested that a potential biological mechanism of cardiovascular disease may occur as a result of release of mediators arising from alveolar inflammation following exposure to ultrafine particles. Fibrinogen and clotting factor VII are plasma proteins involved in blood coagulation. These are important to monitor since they have also been associated with increased ischemic heart disease (IHD) (Seaton *et al.*, 1995; Sweetnam *et al.*, 1996; Levenson *et al.*, 1995).

Both fibrinogen and clotting factor VII are formed in the liver (Guyton, 1991). As fibrinogen is used in the body, the liver produces more fibrinogen. Release of IL-6 from lung macrophages during an inflammatory response causes the liver to produce more fibrinogen (Seaton *et al.*, 1995), resulting in increased plasma fibrinogen levels. This biofeedback mechanism is necessary for maintenance of homeostasis in the body. In addition, fibrinogen is often elevated during acute exercise due to increased sympathetic nervous system activity (Hall and Adair, 1998).

Blood was collected from each subject pre-exposure, post-exposure, post-exercise and 24-hours post-exposure, as mentioned in Section 4.4.1. The blood was collected in sodium citrate tubes. The samples were centrifuged at 2000 rpm for 20 minutes at -4°C, and plasma samples were obtained. White blood cells were obtained from the sodium citrate tube. All samples were frozen at -70°C for future analyses. Analysis of blood coagulation factors is conducted with citrated plasma since the citrate ions combine with the calcium ions in blood (Guyton, 1991) to prevent blood coagulation prior to analysis.

In Study No. 1, plasma fibrinogen and clotting Factor VII antigen were analyzed at Harvard. Fibrinogen was analyzed by the Clauss (1957) method, using Diagnostica Stago (ST4 Coagulation Instrument, American Bioproduct) to measure clotting time following incubation with calcium thrombin reagent. The normal range for fibrinogen is 200 to 400 mg/dl using this method. Clotting Factor VII antigen was measured using an ELISA enzyme immunoassay kit (Diagnostica Stago, France).

In Study No.2, plasma samples were analyzed for fibrinogen at a local laboratory (St. Michael's Hospital) also using the Clauss (1957) method, adding thrombin to the diluted plasma sample and measuring clotting time. An Ortho Fibrinogen Assay kit was used for this test (Ortho Diagnostic Systems, Inc.). The normal range for fibrinogen values using this method is 130 to 330 mg/dl. The normal range for fibrinogen levels is slightly different using this method than that in Study No.1. Therefore, the fibrinogen values from the two studies will not be directly compared.

4.8 Nasal Lavage

A subtle inflammatory response seen in nasal lavage cellularity may be present following exposure to air pollutants that is not associated with respiratory symptoms or decrements in lung function (Blaski *et al.*, 1996). There can be substantial intra-individual and inter-individual variability with nasal lavage samples (Hauser *et al.*, 1995). Thus, pre-exposure measures for each subject prior to exposure are compared to values post-exposure and 24-hours post-exposure to reduce confounding.

Differential white blood cell counts are performed to assess inflammation at a cellular level. White blood cells are recruited from the bloodstream to areas of inflammation in the body. An increase in inflammatory white blood cells, such as neutrophils, macrophages and eosinophils, are expected following an acute inflammatory reaction. Neutrophils, macrophages and eosinophils phagocytize foreign agents in the body. Eosinophils and basophils are involved in allergic reactions, and lymphocytes are important for immunity (Starr and McMillan, 1997). An acute inflammatory reaction to inhaled particles would result in an increase in white blood cells in respiratory lining fluid. Neutrophils and eosinophils quickly migrate to tissues and macrophages present in the lung tissue mobilize within minutes of the inflammation, but it takes longer for macrophages to be recruited from blood into inflammed tissue (Guyton, 1991).

For the current studies, five ml of sterile phosphate buffered saline (0.9%, pH 7.2), heated to 37°C, was used to perform the nasal lavage with a nasal spray dispenser (Beconase, Glaxo). The subject was instructed to insert the syringe into a nostril as far as

comfortable, tilt their head back, spray approximately 0.5 ml saline, hold for 10 seconds, and blow gently into a sterile collection jar on ice. This was done with each nostril, alternately, with the entire 5 ml of saline. Therefore, the sample was representative of both nostrils. The nasal lavage processing was conducted immediately following collection, according to methods adapted from Koren *et al.* (1990), and described briefly below. The sample was filtered through 48 μm nylon gauze to remove mucus. The filtered sample was centrifuged at 1700 rpm for 20 minutes, and supernatant removed and frozen at -70°C for future analysis of protein concentration and cytokines. The cell pellet was resuspended, weighed, and a cell count performed using the trypan blue exclusion method for cell viability. Total cell counts were performed using a Neubauer hemocytometer at 400x magnification. Cytospins were prepared at 450 rpm for 6 minutes, the slides were air dried, fixed in methanol and stained with Wright-Giemsa stain for differential cell counts. Differential cell counts were obtained with 400 cells.

Protein concentration was measured in nasal lavage fluid as an indicator of inflammation. During an inflammatory response in tissues, there is an increase in vasodilation of local blood vessels, resulting in increased local blood flow. Swelling follows and an increase in capillary permeability results in increased leakage of plasma proteins into interstitial spaces (Guyton, 1991). This increase in permeability can be measured with increased protein in airway fluid. The body automatically blocks off the area of inflammation by formation of fibrin clots to stop loss of fluid through the spaces. The reaction is intensified with increased injury. Nasal lavage supernatant samples were analyzed for protein concentration using a protein assay kit from Pierce.

4.9 Sputum Induction

Sputum has been measured in many studies to identify whether there is inflammation at a cellular level. Sputum samples are reflective of inflammation in the airways and it is noted that the cells obtained are from the bronchi and bronchioles, but not from the alveoli. Sputum induction is reliable since it is reproducible, and can be performed on the same subject a number of times (Pizzichini *et al.*, 1996). The rationale for the differential cell count in sputum is the same as for nasal lavage, presented above.

Sputum was induced through inhalation of aerosolized hypertonic saline using a Medix ultrasonic nebulizer. Saline concentrations in order of 3%, 4% and 5% were inhaled for 7 minutes each, and the subjects were instructed to cough from deep in their lungs following each inhalation, after blowing their nose and rinsing with water to minimize upper airway contamination. The induced sputum was collected in a sterile container on ice and processed immediately using the technique of Pizzichini *et al.* (1996). Mucus portions of the sample were selected to minimize squamous cell contamination from saliva. The lack of squamous cell contamination (<10%) was confirmed under a microscope (magnification 100x) prior to processing of the sample. The sample was treated with dithiothreitol (DTT) (Sputolysin reagent, Calbiochem-Novabiochem Corp., San Diego California) for 15 minutes to breakdown the mucus. The sample was then diluted with phosphate buffered saline (PBS) (Gibco BRL, Life Technologies, Grand Island, New York) and filtered through 48 μm nylon gauze to remove cell debris and mucus. The trypan blue exclusion method was used to determine cell viability, and total cell counts were performed using a Neubauer hemocytometer at 400x magnification.

Cytospins were prepared at 450 rpm for 6 minutes (using the original filtrate, adjusted to equal approximately 1,000,000 cells/mL). The slides were air dried, fixed in methanol and stained with Wright-Giemsa stain for differential cell counts with 400 cells. The sample was then centrifuged at 1700 rpm for 20 minutes, supernatant obtained and frozen at -70°C then archived for possible future cytokine analyses.

Prior to processing of the induced sputum sample, some of the raw sputum samples were frozen at -70°C for analysis of protein concentration using a protein assay kit from Pierce. The rationale for analyzing for protein concentration is given above in Section 4.8. The protein assay kit used is affected by DTT, and therefore the supernatant from the sputum samples cannot be used for this assay. The raw sputum samples were analyzed for protein in triplicate, and an average value was obtained for each sample due to the heterogeneous nature of raw sputum.

4.10 Statistics

Statistical analyses were not conducted on the outcome variables from either study due to limitations with the small subject number and a number of missing or inadequate samples for sputum, nasal lavage fluid and blood coagulation factors. This was further amplified in Study No. 2 due to subject attrition late in the study after completion of 2 pollutant exposures but no FA exposure. For these reasons, and following discussion with a biostatistician (P. Corey, personal communication), data from individuals were analyzed for trends to increase or decrease following exposures. Some data, such as particle mass and gases, are presented as mean levels to simplify presentation of the data.

5.0 RESULTS FROM CAP EXPOSURES – STUDY NO. 1

Exposure Concentrations: The average ambient $PM_{2.5}$ mass concentration (gravimetric) outdoors during exposures was 22 ± 14 ranging from 5 to 45 $\mu g/m^3$. Based on filter mass, the respective means (± SD) for the presumed low, medium and high CAP categories were 31.5 ± 7.9, 52.9 ± 33.9 and 92.1 ± 24.6 $\mu g/m^3$. These levels are reasonably close to, but higher than the levels of 20, 40, and 60 $\mu g/m^3$, which were the targets using the real-time DustTrak. Mean gas concentrations measured during exposures were slightly lower in the CAP air stream compared to ambient, with no significant differences in mean concentration between the CAP exposures and FA. Mean gas concentrations during CAP exposures were 9 ± 8 ppb O_3, 18 ± 19 ppb NO, 20 ± 7 ppb NO_2, 0.3 ± 0.3 ppm CO and 2.3 ± 3.3 ppb SO_2. These were very similar to the gas concentrations during FA exposures, suggesting no confounding for gaseous pollutants during exposures. Mean concentrations of environmental variables are presented in Table 5-1. The individual data for $PM_{2.5}$ and gaseous pollutants are presented in Appendix A, Table A-1. Mean levels of $PM_{2.5}$ and gases for exposures are similar to mean Toronto ambient levels (Section 1).

Table 5-1: Mean (±SD) PM & Gases in HEF During Exposure for Healthy Subjects

Environmental Variables	FA	Low CAP	Mid CAP	High CAP
$PM_{2.5}$ gravimetric ($\mu g/m^3$)	na	31.5 ± 7.9	52.9 ± 33.9	92.1 ± 24.6
Ozone (ppb)	9.3 ± 11.6	10.9 ± 9.5	10.5 ± 10.3	7.0 ± 2.6
Carbon monoxide (ppm)	na	0.2 ± 0.1	0.3 ± 0.2	0.5 ± 0.4
Nitrogen dioxide (ppb)	na	17.4 ± 9.2	18.8 ± 7.9	25.2 ± 5.5
Nitric oxide (ppb)	9.1 ± 7.7	13.4 ± 9.1	16.8 ± 14.2	32.1 ± 30.6
Sulfur dioxide (ppb)	5.6 ± 4.9	0.6 ± 0.4	2.4 ± 3.2	2.0 ± 1.3

Blood Coagulation Activity: The percent change in fibrinogen levels post-exercise showed a trend to show a greater increase above the pre-exposure levels at the High CAP compared to FA in three of four subjects (Figure 5-1). The increases were greatest in the two female subjects (PS2 and PS3). The actual values are shown in Table 5-2.

There was no apparent dose-response based on target mass CAP concentrations. Fibrinogen levels in samples taken immediately after exposure and 24-hours post-exposure did not appear to differ with CAP or FA in each individual (Table A-2). Since fibrinogen is an acute-phase protein, and is expected to increase following inflammation, the increase at approximately 3 hours post-exposure (e.g., following exercise with High CAP vs. FA) may be considered physiologically relevant. There did not appear to be any treatment-related effects on clotting factor VII antigen levels at any time points, as shown in Table A-2.

Table 5-2: Plasma Fibrinogen Concentrations Post-Exercise in Healthy Subjects

	Fibrinogen (mg/dl)			
Subject/Sample	**FA**	**Low CAP**	**Mid CAP**	**High CAP**
PS2				
Pre-exposure	340	348	313	350
Post-exercise	358	363	303	406
PS3				
Pre-exposure	295	312	263	368
Post-exercise	313	313	274	439
PS5				
Pre-exposure	229	236	216	197
Post-exercise	226	225	206	206
PS7				
Pre-exposure	222	224	221	230
Post-exercise	219	215	232	226

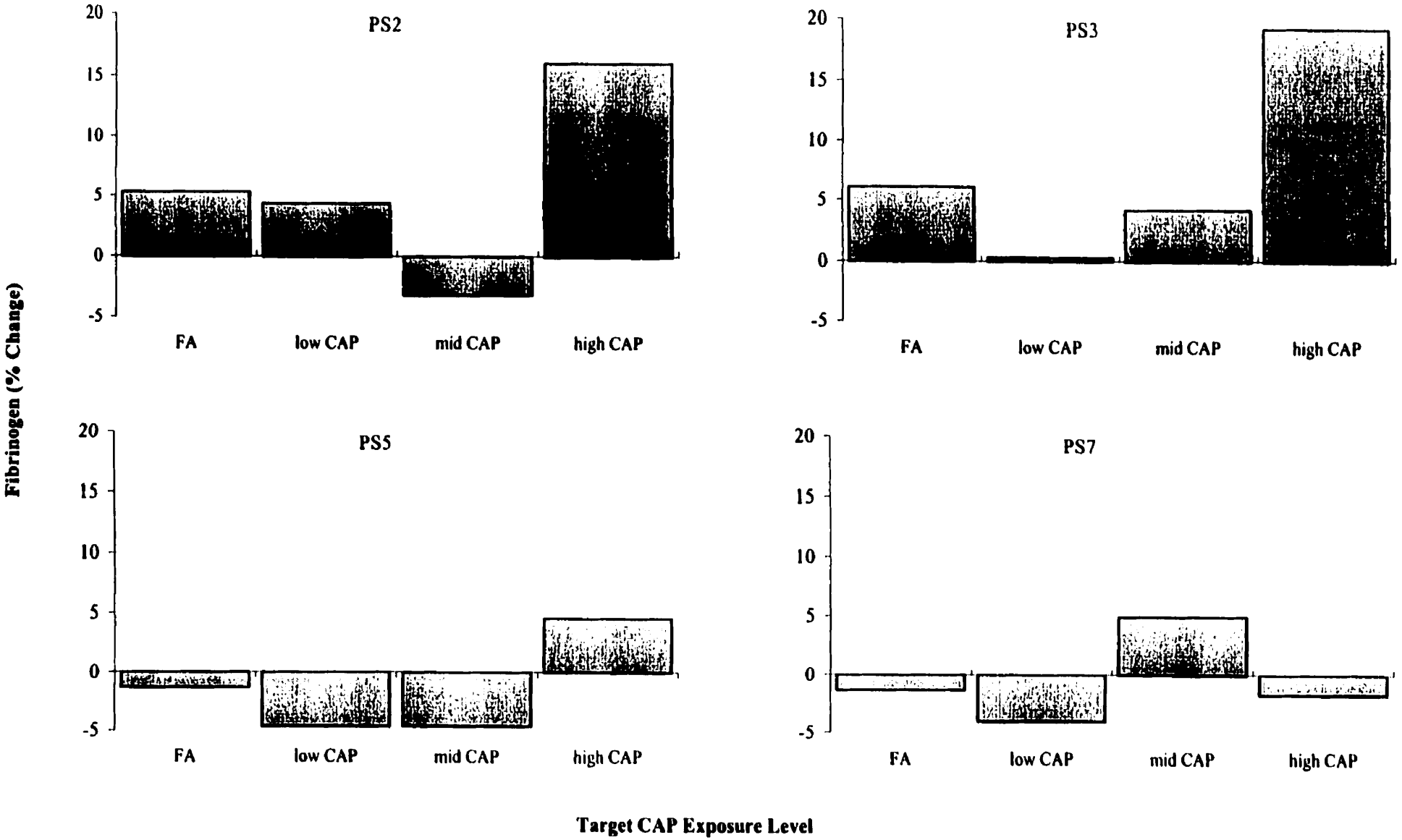

Figure 5-1: Percentage Change in Plasma Fibrinogen Levels Post-exercise vs. Pre-exposure

Note: percentage change = 100*((post-exercise-pre-exposure)/pre-exposure); Data from FA compared with CAP exposures

ECG/Holter: The 12-lead ECG printouts obtained during FA and CAP exposures and the following exercise periods were assessed as normal by a cardiologist when reviewed for abnormalities in ECG waveforms. Some ectopic activity was noted; however, it is normal for healthy adults to have some ectopic heartbeats (Kamath and Fallen, 1995). There was no apparent effect on the ECG (e.g., ectopic heartbeats) associated with CAP vs. FA exposures. ECG printouts cannot be included as part of this thesis for review due to the copious amount of data. Following each exposure, the ECG printouts were reviewed by a cardiologist to confirm that there were no clinically significant findings. Also, blood pressure measurements taken during exercise did not appear to be affected by CAP exposure.

Preliminary analyses of Holter tapes from Harvard showed no trends to decreased HRV as assessed by SDNN, over the two-hour CAP exposures compared to FA (Figure 5-2). Results for each subject are shown in Table A-3 for exposure and Table A-4 for exercise. No consistent dose-response trends were seen with any HRV parameters during the exposure or exercise periods for the subjects.

Nasal Lavage: We obtained nasal lavage fluid samples for all subjects prior to exposure, post-exposure and 24-hours post-exposure. Differential cell counts could not be obtained on some of the samples for a variety of reasons. Some of the samples were too mucoid to be filtered sufficiently for processing, or the samples were heavily contaminated with red blood cells or the white blood cells were damaged during processing, thus preventing an accurate differential cell count. Data were processed only for slides that were of good quality. Average values for each of the parameters are summarized in Table 5-3. Data for

each individual are presented in Table A-5. Due to the high inter-individual variability seen, the data are discussed as increases or decreases from pre-exposure values.

Table 5-3: Mean (± SD) Values for Nasal Lavage Total Cell Counts and Percent Differentials in Healthy Subjects

Target Exposure	Sampling Time	Cell Viability (%)	Total cell count (10^3 cells/ml)	Epithelial Cells (%)	Neutrophils (%)	Macrophages (%)	Eosinophils (%)
FA	Pre-exposure	67 ± 18	322 ± 252	66 ± 26	31 ± 24	0.4 ± 0.4	1.5 ± 2.2
	Post-exposure	66 ± 15	380 ± 194	63 ± 22	32 ± 21	1.0 ± 0.4	2.1 ± 3.8
	24 hr post	58 ± 16	441 ± 452	71 ± 15	27 ± 14	0.7 ± 0.1	0.9 ± 1.0
Low CAP	Pre-exposure	62 ± 11	462 ± 405	83 ± 12	14 ± 11	0.3 ± 0.3	0.4 ± 0.4
	Post-exposure	71 ± 17	1199 ± 1606	51 ± 25	45 ± 23	1.1 ± 1.2	1.0 ± 1.0
	24 hr post	57 ± 12	438 ± 491	41 ± 21	53 ± 16	1.1 ± 0.9	3.9 ± 6.6
Mid CAP	Pre-exposure	53 ± 14	964 ± 651	70 ± 18	26 ± 17	0.8 ± 0.8	2.4 ± 4.4
	Post-exposure	66 ± 19	511 ± 492	51 ± 26	46 ± 24	0.7 ± 0.7	1.2 ± 1.9
	24 hr post	64 ± 16	808 ± 556	60 ± 13	36 ± 14	1.4 ± 0.6	1.1 ± 1.9
High CAP	Pre-exposure	54 ± 24	1033 ± 959	47 ± 20	50 ± 21	0.3 ± 0.0	1.2 ± 0.1
	Post-exposure	52 ± 14	704 ± 220	62 ± 23	37 ± 23	0.4 ± 0.5	0.8 ± 0.9
	24 hr post	51 ± 16	719 ± 396	34 ± 11	62 ± 9	1.2 ± 0.3	1.9 ± 1.7

In addition to the inter-individual variability, high intra-individual variability was found with nasal lavage total cell counts and differentials. There were no apparent treatment-related effects on total cell counts, eosinophils, lymphocytes or cell viability. Figures 5-3 to 5-5 show nasal lavage total cell counts, number of neutrophils, and number of macrophages pre-exposure, post-exposure and 24-hours post-exposure. There appeared to be an increase in macrophages 24-hours post-exposure at the mid- and high CAP compared to FA exposures. For pooled CAP exposures there was an increase (above the pre-exposure value) in the percentage of neutrophils measured approximately two hours after exposure (post) for 7/10, or 70% of observations, and 8/9, or 89% of observations,

24-hr after exposure (24-hr post). Only one subject (PS7) showed an increase in neutrophils with FA, at both time points.

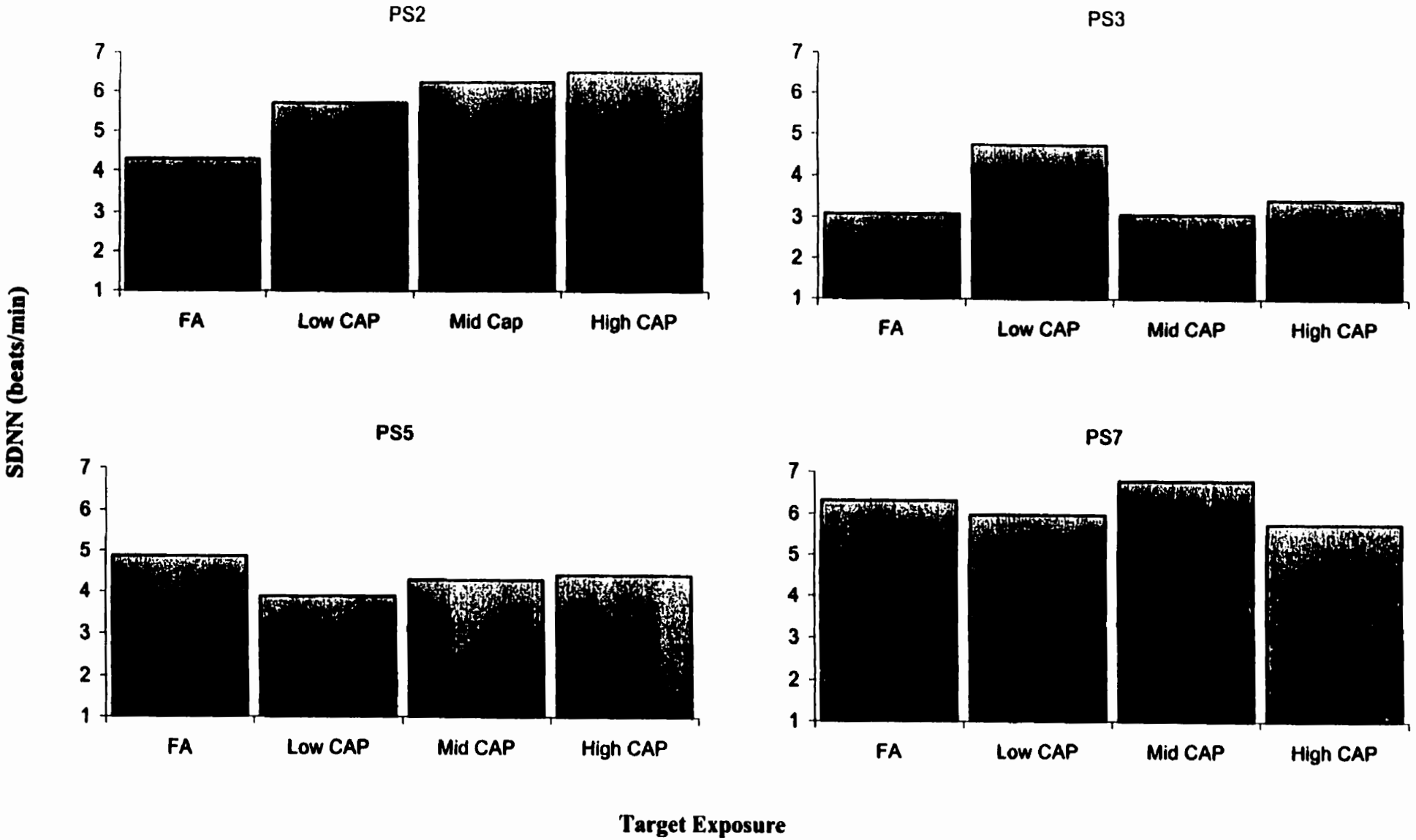

Figure 5-2: SDNN During Exposure in Healthy Subjects

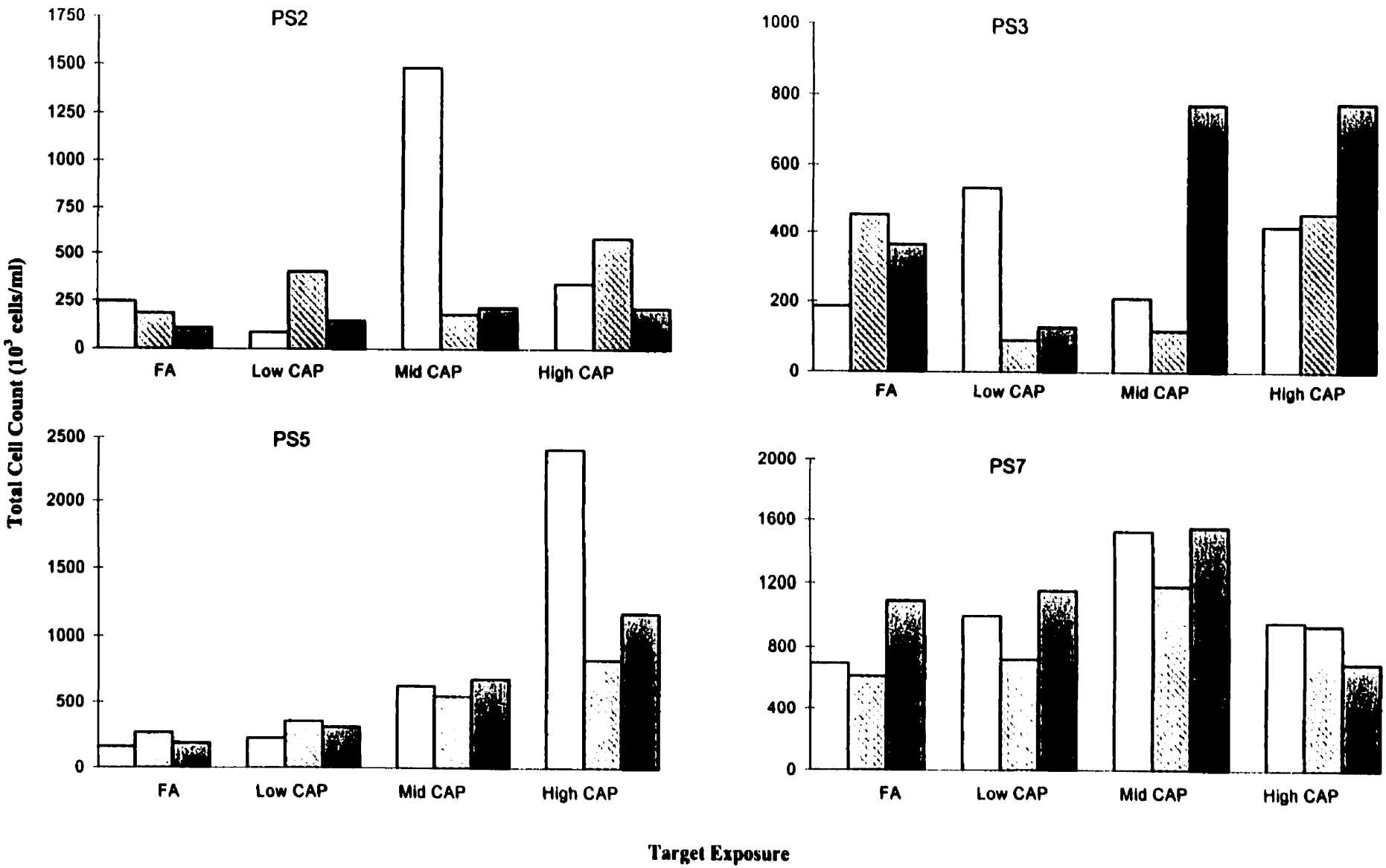

Figure 5-3: Nasal Lavage - Total Cell Counts in Healthy Subjects Pre-exposure, Post-exposure and 24-hours Post-exposure

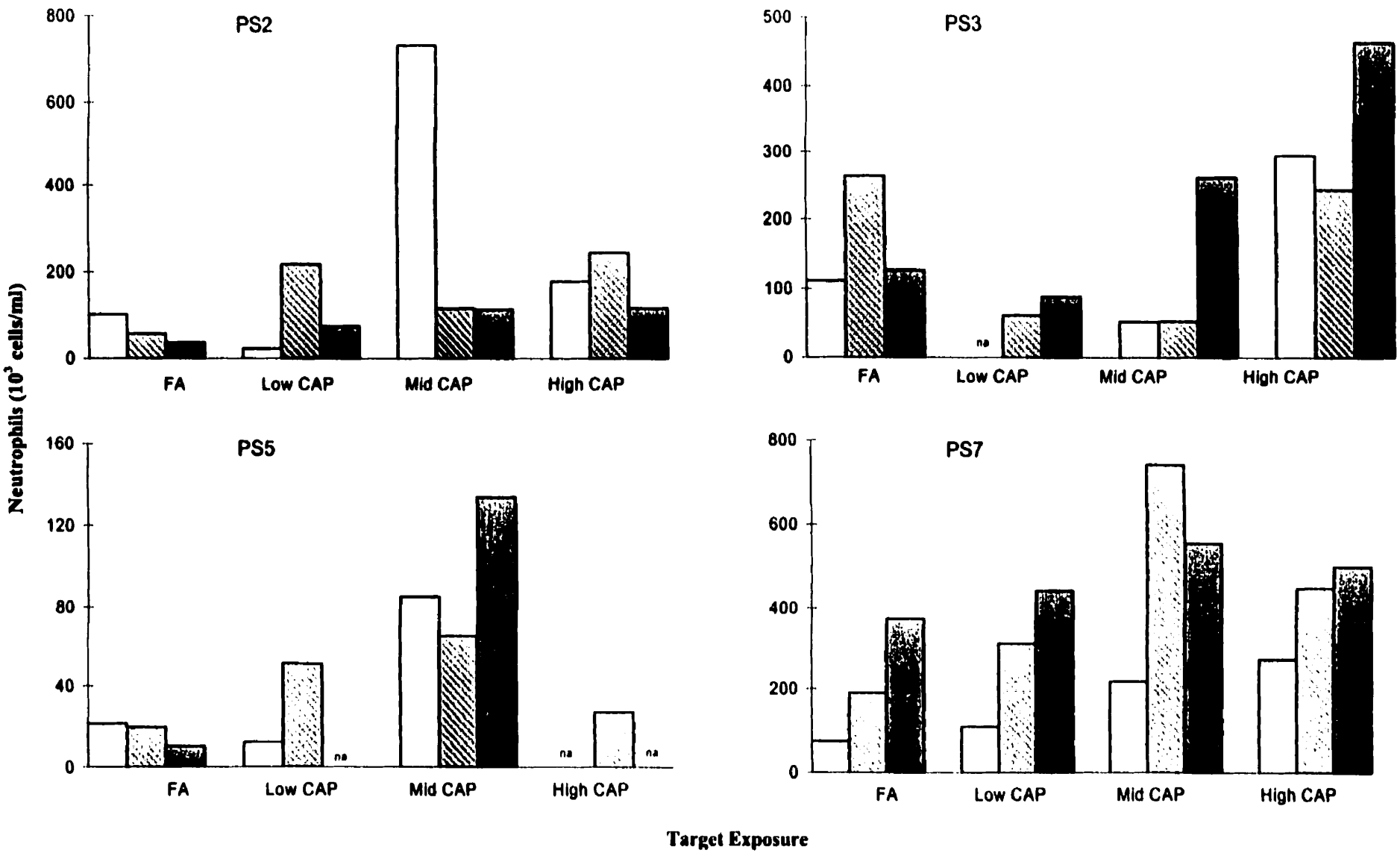

Figure 5-4: Nasal Lavage - Neutrophils in Healthy Subjects Pre-exposure, Post-exposure and 24-hours Post-exposure

pre-exposure post-exposure 24-hours post-exposure

na = no differential cell count available

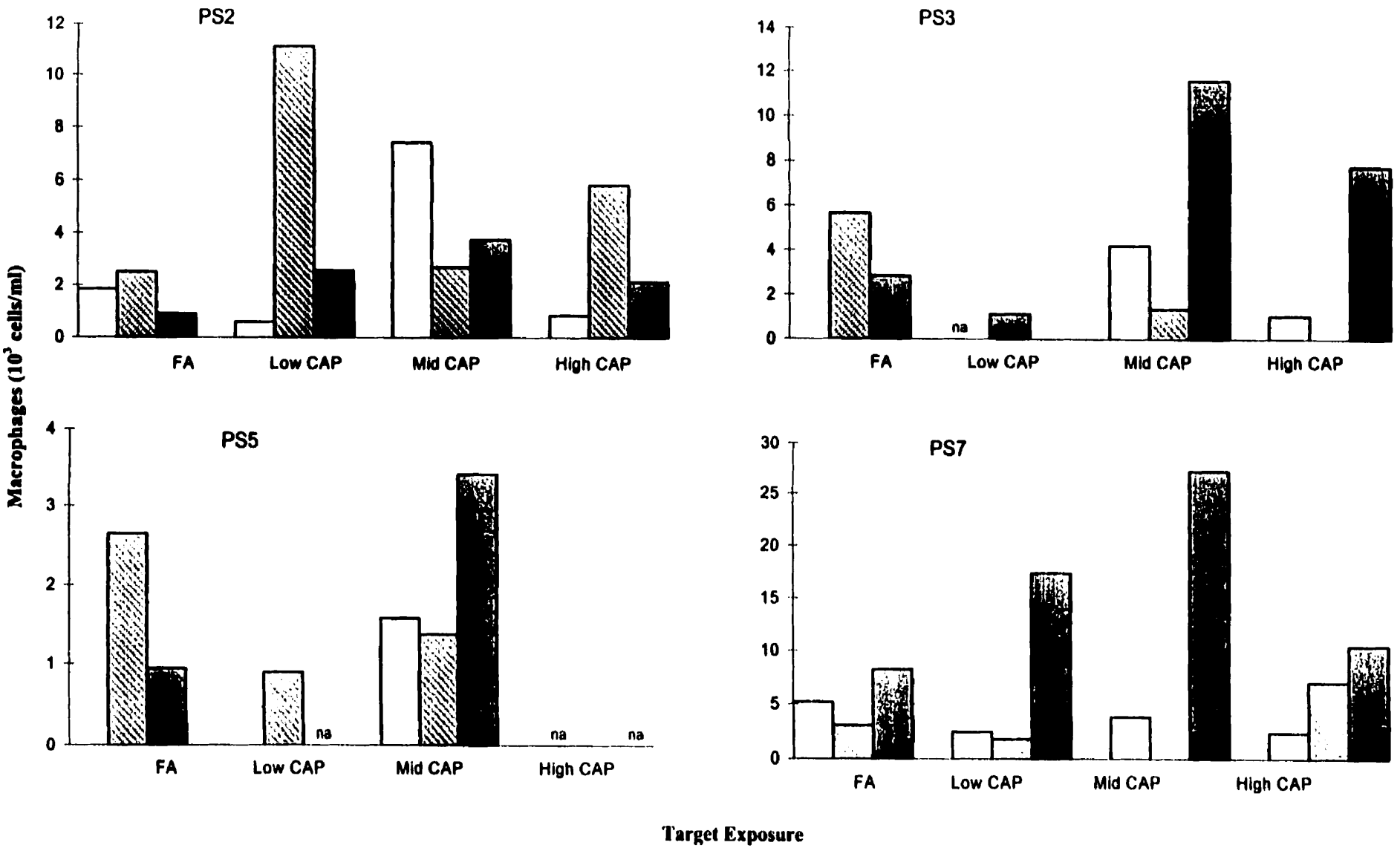

Figure 5-5: Nasal Lavage - Macrophages in Healthy Subjects Pre-exposure, Post-exposure and 24-hours Post-exposure

pre-exposure post-exposure 24-hours post-exposure

na = no differential cell count available

Protein content in nasal lavage fluid supernatant did not show trends to increase with CAP vs. FA either post-exposure or 24-hours post-exposure (see Table 5-4). Data for individuals are presented in Table A-6.

Table 5-4: Mean (± SD) Protein Concentration in Nasal Lavage Fluid and Raw Sputum in Healthy Subjects

Target CAP Exposure	Sampling Time	Nasal Lavage Protein Concentration (μg/ml)	Raw Sputum Protein Concentration (μg/ml)
FA	pre-exposure	543 ± 190	na
	post-exposure	594 ± 217	1872 ± 694
	24-hr post-exposure	673 ± 325	2236 ± 1031
Low CAP	pre-exposure	584 ± 134	na
	post-exposure	638 ± 160	2227 ± 1235
	24-hr post-exposure	831 ± 71	2986 ± 1720
Mid CAP	pre-exposure	654 ± 81	na
	post-exposure	652 ± 213	2865 ± 1959
	24-hr post-exposure	732 ± 138	2804 ± 1259
High CAP	pre-exposure	567 ± 310	na
	post-exposure	611 ± 267	2086 ± 265
	24-hr post-exposure	675 ± 308	2561 ± 230

na = no pre-exposure sputum samples taken in Study No. 1

Sputum: As discussed in the methods, samples for sputum were obtained post-exposure and 24-hours post-exposure. In Study No. 1, no sputum samples were obtained pre-exposure. We were not able to obtain sufficient sample for processing for five CAP sampling points and therefore had a smaller sample set than anticipated. There appeared to be a lot of amount of intra-individual and inter-individual variability in sputum samples. This may confound interpretation of data since it is not known what baseline values are for each day pre-exposure. Data for CAP exposures were compared with FA

exposures. Total protein concentrations in raw sputum did not appear to differ following CAP vs. FA exposures (see Table 5-4). Individual data are shown in Table A-6.

There was no strong trend to a decrease in cell viability in raw sputum samples with CAP exposure compared with FA, although it is difficult to assess due to the number of samples that could not be analyzed, resulting in a smaller data set. Similarly, there were no trends with total cell counts, percent differentials or epithelial cells post-exposure or 24-hours post-exposure. Total cell counts and percent neutrophils for individuals are presented in Table 5-5. Additionally, percent neutrophils for individuals post-exposure are shown in Figure 5-6. The individual data for cell viability, total cell counts, percent differentials and epithelial cells are shown in Table A-7.

Table 5-5: Sputum Total Cell Counts and Percent Neutrophils in Healthy Subjects

Subject	Target CAP Exposure	Total cell count (10^3 cells/ml)		Neutrophils (%)	
		post-exposure	24-hour post-exposure	Post-exposure	24-hour post-exposure
PS2	FA	313	564	75.00	66.00
	Low CAP	831	580	20.50	30.75
	Mid CAP	476	675	43.50	50.25
	High CAP	456	411	46.25	54.75
PS3	FA	404	187	9.00	20.75
	Low CAP	227	na	7.00	na
	Mid CAP	196	na	na	na
	High CAP	na	na	na	na
PS5	FA	1100	536	81.00	70.75
	Low CAP	620	8206	62.75	84.75
	Mid CAP	1220	1300	44.25	69.25
	High CAP	855	na	24.75	na
PS7	FA	811	951	14.50	27.75
	Low CAP	1015	1175	20.00	43.25
	Mid CAP	744	904	22.75	37.25
	High CAP	984	544	23.50	40.25

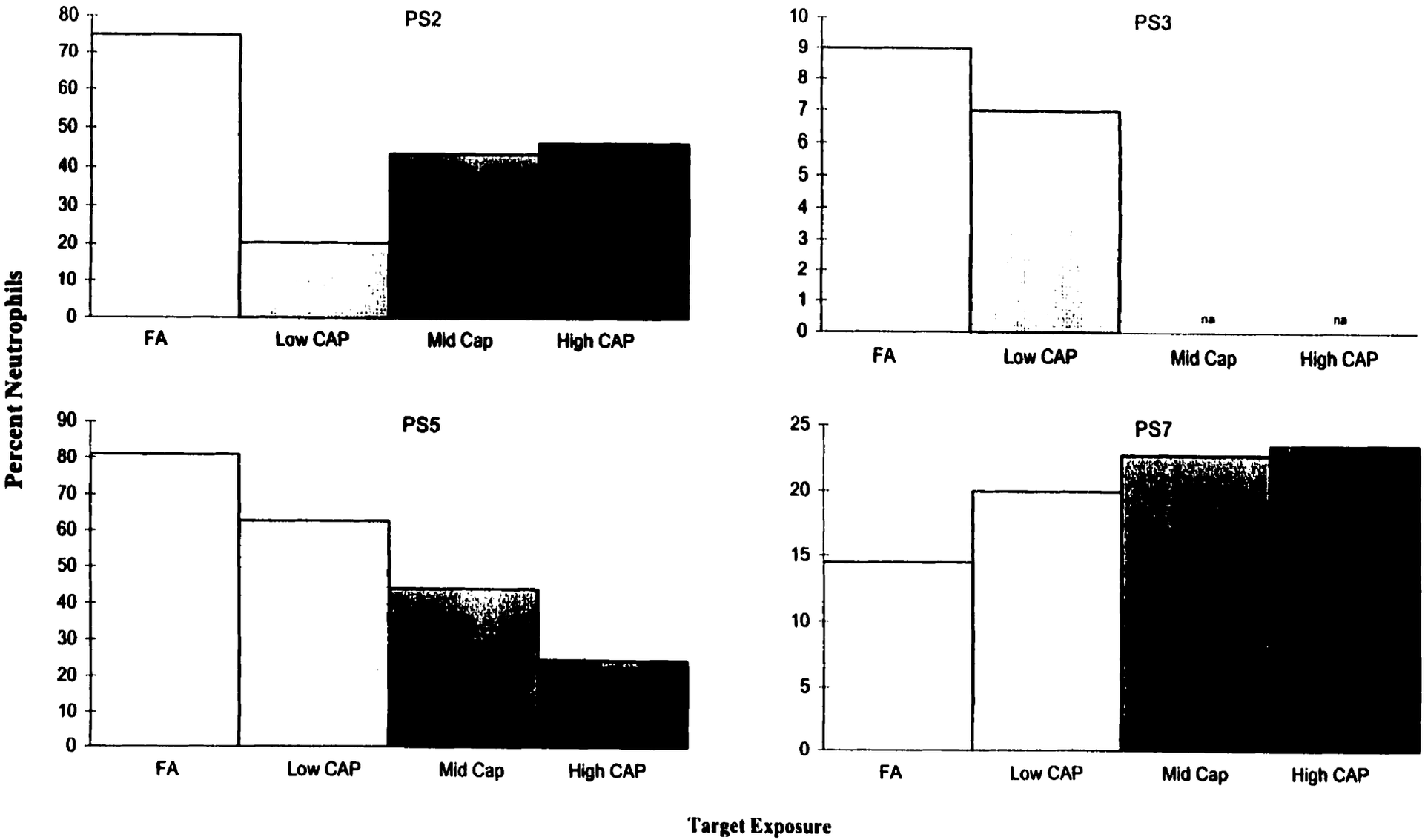

Figure 5-6: Percent Neutrophils in Sputum of Healthy Subjects Post-exposure

na = No differential cell count available

To summarize our results from young, healthy subjects, there were no clinically significant cardiac effects evident from ECG waveforms or HRV. Additionally, there was no strong inflammatory response evident in the respiratory tract of subjects exposed to high CAP, with the exception of a trend to increased neutrophils in nasal lavage fluid 24-hours post-exposure. The acute, reversible trend to increase in plasma fibrinogen levels at the high CAP exposure was of interest. Based on these results, it was decided that Study No. 2 should proceed using the high CAP level of exposure in a more sensitive group of individuals, mild asthmatic adults, who are expected to have a stronger response to CAP due to their asthmatic condition. Additionally, it was decided that at this time, the effects of combined exposure to ozone and CAP could be studied safely. We elected to continue to study levels of pollutants at realistic levels indicative of smoggy days in Toronto.

6.0 RESULTS FROM CAP/OZONE EXPOSURES – STUDY NO. 2

Exposure Concentrations: In Study No. 2, exposures to CAP were monitored in the same fashion as for Study No. 1 using the DustTrak and gravimetric methods. Mean CAP mass concentrations during exposure from filter samples are shown in Table 6-1 with concentrations of gaseous pollutants. The measured ambient gaseous pollutants in the HEF were similar for most of the exposures. For Study No. 2, the correction factors for the DustTrak used to estimate the target filter mass concentrations were varied for each exposure. The DustTrak was calibrated the day prior to each exposure to obtain filter mass concentrations closer to target values. In order to do this, each day prior to exposure, a filter sample was obtained to obtain a correction factor with the DustTrak (measured in the CAP airstream). The factor of DustTrak/filter mass concentration was used to determine the target DustTrak concentration for the exposure. This assumed there would be similar environmental conditions (and therefore particle composition) for the following exposure day.

Table 6-1: Mean (±SD) $PM_{2.5}$ and Gases in HEF During Exposure for Asthmatics

Target Exposure:	FA	CAP	O_3	CAP+O_3
Filter mass concentration ($\mu g/m^3$)	1.2±1.2	46.7±10.3	3.9±2.6	80.9±38.2
Ozone (ppb)	16.2±12.4	2.9±2.2	80.4±6.1	83.2±0.4
Carbon monoxide (ppm)	0.7±0.3	1.0±0.4	0.8±0.2	0.6±0.2
Nitrogen dioxide (ppb)	19.7±3.9	na	na	26.3±6.2
Nitric oxide (ppb)	17.8±13.7	na	na	na
Sulfur dioxide (ppb)	2.6±2.2	3.6±2.7	3.3±2.1	1.5±1.0

na = insufficient data to provide a mean (e.g., n<3) ; Max CAP not shown since n=2; Note: Instrumentation not working during some exposures.

As discussed in Section 4, the target mass CAP concentration was 0 $\mu g/m^3$ for FA, 60 $\mu g/m^3$ for CAP and CAP+O_3, and 120 $\mu g/m^3$ for Max CAP. The particulate mass concentrations varied for each individual exposure, with lower concentrations reached for the CAP and higher concentrations reached for the CAP+O_3 on average. Although the CAP concentrations were variable, comparisons between groups are possible qualitatively since the CAP concentrations were relatively close to target. The target concentration for O_3 of 80 ppb was achieved in the exposures. The gaseous pollutant concentrations during exposures were within the range of background for Toronto ambient air. Results of particle and gaseous pollutant exposure concentrations for individual exposures are shown in Table B-1.

Blood Coagulation Activity: Plasma samples were obtained for all subjects. However, there were numerous missing data points for fibrinogen in this study. This was largely due to the presence of small blood clots in several plasma samples (Table B-2). In addition to this, as noted in Section 4, one of the subjects, PO5, dropped out of the study after completing only two exposures (CAP and O_3), resulting in fewer data points than planned in the original protocol for the study. The study design was changed to include a Max CAP exposure, but only two subjects completed this exposure. These factors resulted in insufficient data to provide mean values for fibrinogen.

No trends for fibrinogen concentrations were evident with pollutant exposures compared with FA exposures at any time points sampled. Figures 6-1 and 6-2 show the percent change in plasma fibrinogen post-exposure and post-exercise.

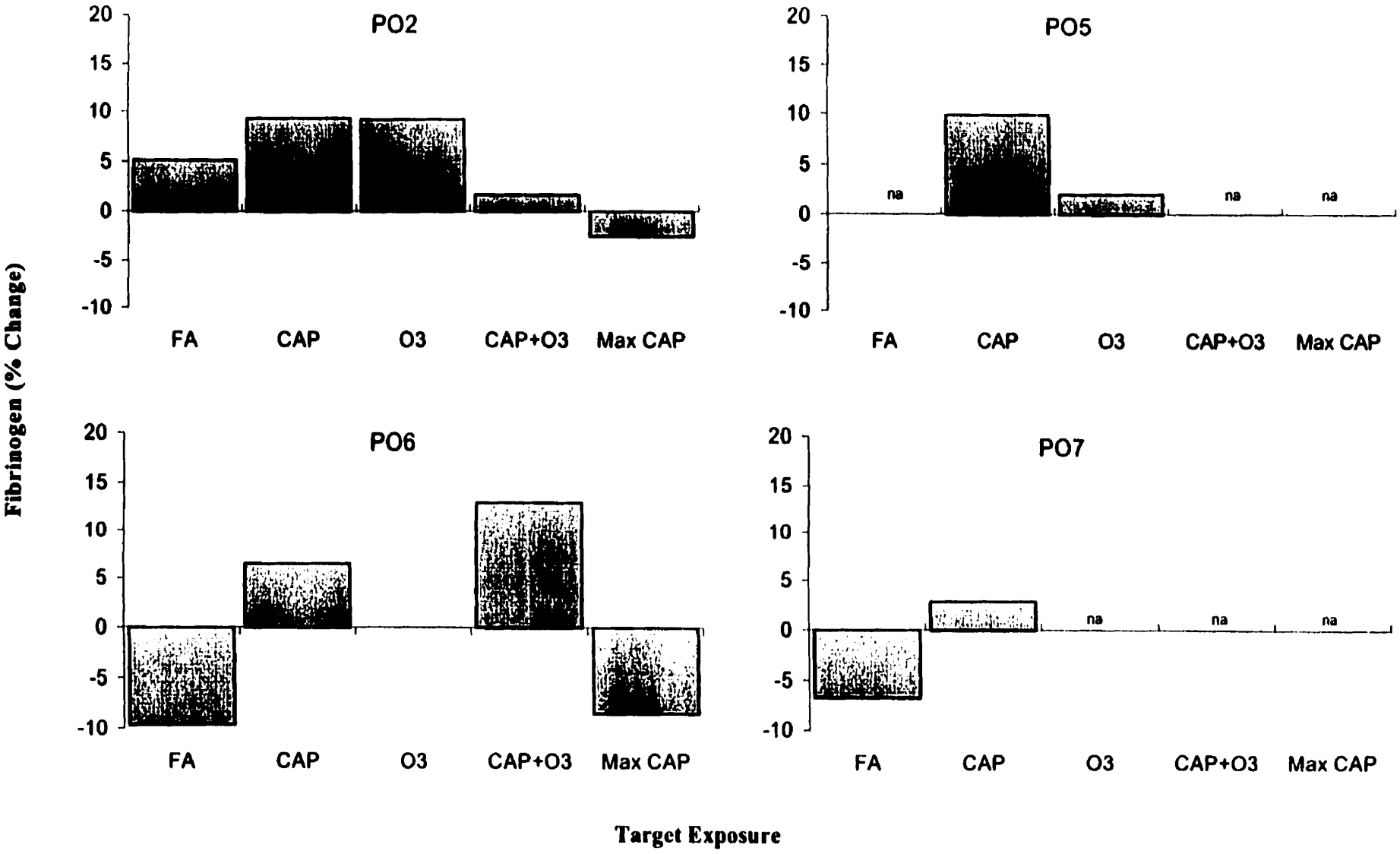

Figure 6-1: Percent Change in Plasma Fibrinogen Levels Post-exposure vs. Pre-exposure

Note: percent change = 100*((post-exposure-pre-exposure)/pre-exposure); Data from FA compared with pollutant exposures
na = data not available

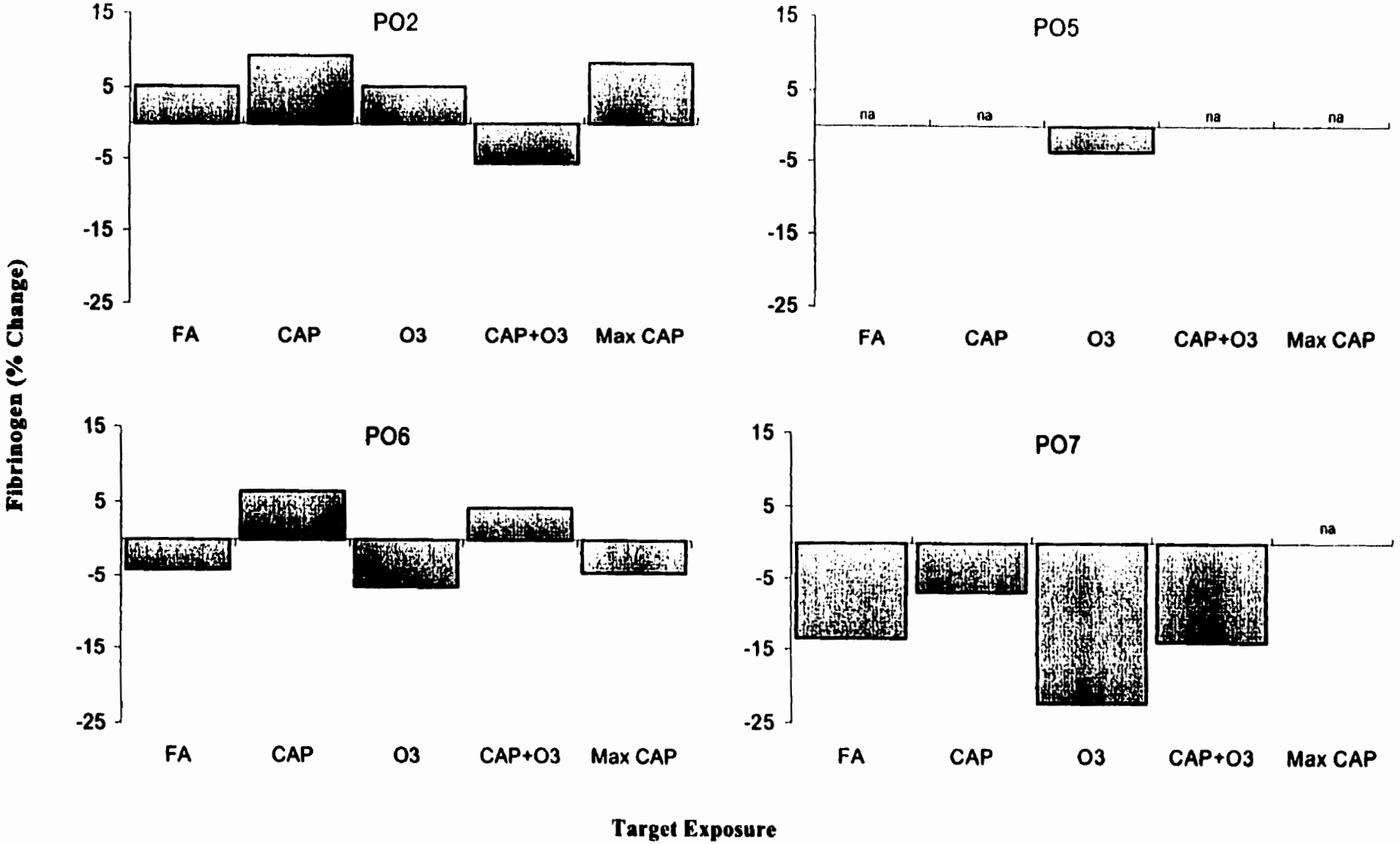

Figure 6-2: Percent Change in Plasma Fibrinogen Levels Post-exercise vs. Pre-exposure

Note: percent change = 100*((post-exercise-pre-exposure)/pre-exposure); Data from FA compared with pollutant exposures
na = data not available

ECG/HRV: ECG waveforms were normal for all exposures in this study, as assessed by a cardiologist. There were no treatment-related changes apparent in ECGs during any of the time points studied, including exposure and exercise.

HRV analyses were conducted for all but two exposures. For one subject, PO5, a large portion of the CAP exposure period was not monitored due to a defective tape. Therefore the data could not be analyzed for this exposure. Similarly, there were no Holter data for the Max CAP exposure for PO6, also due to a defective tape. As mentioned in Section 4, analyses were conducted for LF power, HF power and LF/HF ratio, SDNN, rMSDD and pNN50. Since it is necessary to compare HRV for intervals of the same duration, exposure and exercise data for pollutant exposures were compared against FA values at the same time points. Figures 6-3 and 6-4 show results for SDNN and HF Power, respectively. Individual results for HRV parameters are shown in Tables B-3 for exposure and B-4 for exercise.

The HRV did not show any strong dose-related trends during pollutant exposures or exercise compared with FA. No strong pattern of response was evident with mean heart rate (HR) during exposure. Similarly, there were no trends in HF power, indicative of vagal activity, with pollutant vs. FA exposures (Figure 6-4). This was consistent with rMSDD and pNN50 parameters, also indicative of vagal activity (Table B-3). Due to a number of missing data points for these parameters, resulting from defective tapes and subject attrition, it is not possible to make any firm conclusions.

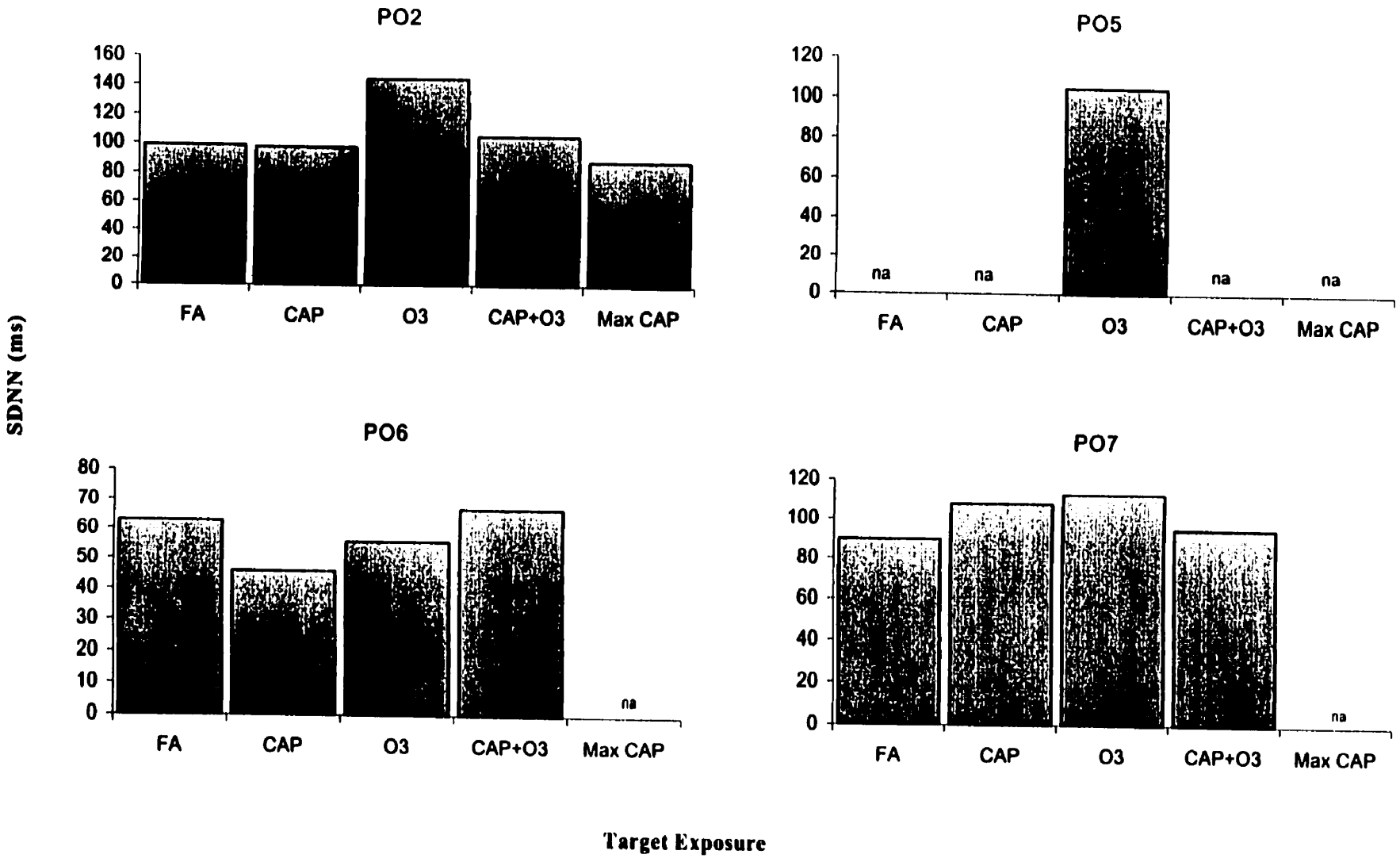

Figure 6-3: SDNN During Exposure in Asthmatic Subjects
na = data not available

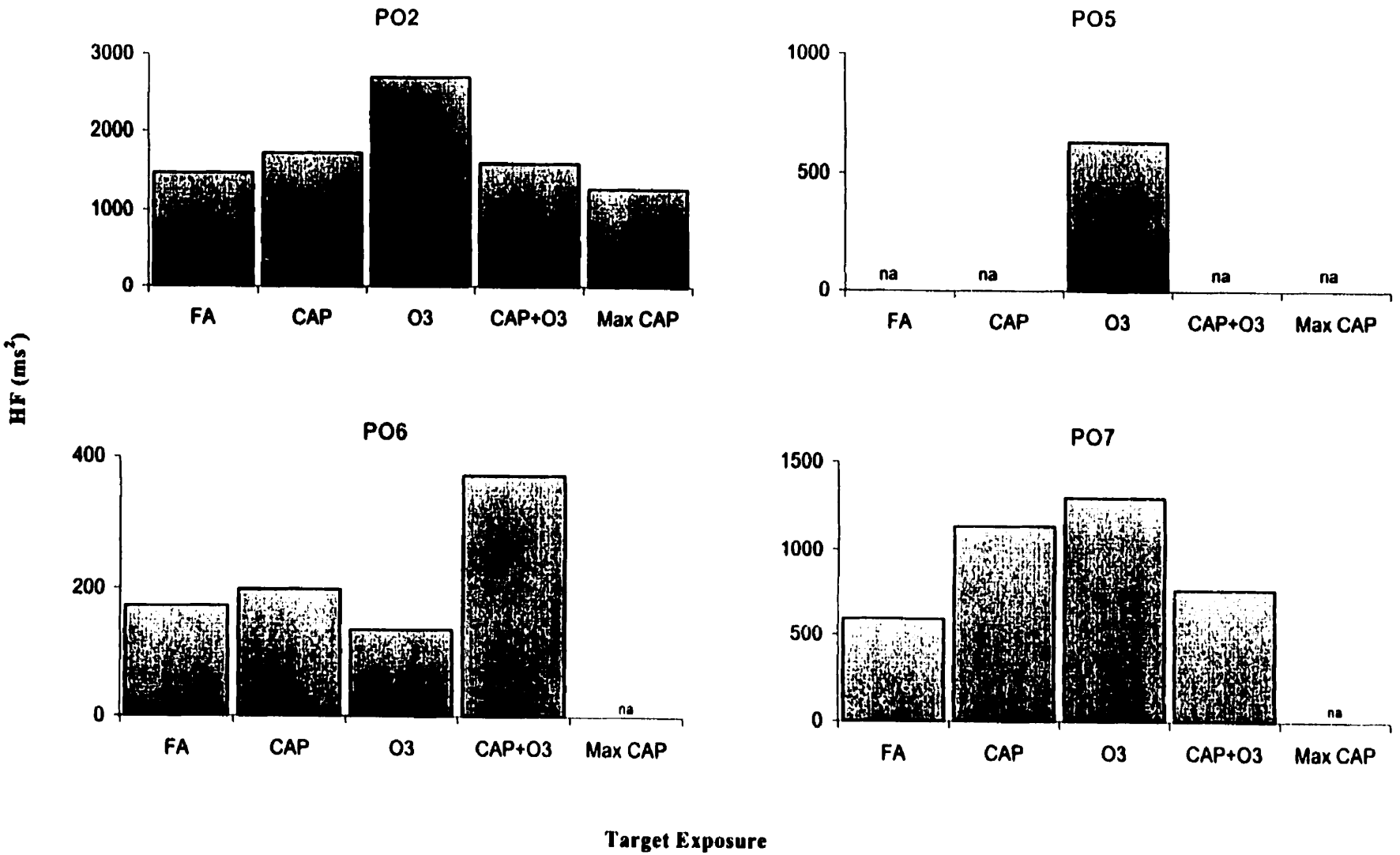

Figure 6-4: High Frequency Power During Exposure in Asthmatic Subjects
na = data not available

Nasal Lavage: Similar to the results from Study No.1, differential cell counts were not always possible to obtain due to the presence of numerous red blood cells or damaged white blood cells. There were five missing samples, including four pre-exposure values, out of a total of 48 sampling time points. Mean values for nasal lavage fluid parameters are shown in Table 6-2. It is not possible to statistically analyze the data due to the large number of missing data points. Data for the Max CAP exposure are not shown in Table 6-2 since there were only two subjects for this exposure and therefore no mean was obtained. Individual data are provided in Table B-5. The available data do not show a strong trend indicative of inflammatory response (e.g., no consistent increase in neutrophils) for the pollutant exposures compared with FA.

Table 6-2: Mean (±SD) Values for Nasal Lavage Total Cell Counts and Percent Differentials in Asthmatic Subjects

Target Exposure	Sampling Time	Cell Viability (%)	Total cell count (10^3 cells/ml)	Epithelial Cells (%)	Neutrophils (%)	Macrophages (%)	Eosinophils (%)
FA	pre-exposure	66±8	221±227	na	na	na	na
	post-exposure	65±11	531±408	32±23	62±20	0.4±0.5	4.8±4.2
	24 hr post	82±6	200±51	na	58±19	0.4±0.3	8.9±6.8
CAP	pre-exposure	69±18	684±575	31±41	65±36	0.3±0.4	3.0±4.2
	post-exposure	68±24	473±675	9±5	76±25	0.6±0.6	11±17
	24 hr post	82±16	519±327	21±28	70±29	0.8±0.4	12±7
O_3	pre-exposure	70±28	518±263	45±38	51±38	0.5±0.4	2±0.2
	post-exposure	76±11	443±326	41±10	53±9	0.4±0.3	5±3
	24 hr post	84±16	632±581	21±14	59±12	0.3±0.5	21±21
CAP+O_3	pre-exposure	81±13	688±597	na	na	na	na
	post-exposure	82±4	484±390	10±8	87±5	0±0	2.1±2.8
	24 hr post	77±16	778±555	na	60±23	1.3±1.4	10±17

na = insufficient data to provide a mean (e.g., n<3); Max CAP not shown since n=2

In Figure 6-5 it can be seen that in subject PO2 there was an increase in percent eosinophils following CAP, Max CAP and O_3 compared to pre-exposure, but not with CAP+O_3. In subject PO5, there was an increase in eosinophils following exposure to O_3, but there was no FA exposure for this subject to use as a control. PO6 showed an increase with Max CAP, and PO7 showed an increase with O_3. No trends were seen with total cell counts (Figure 6-6), neutrophils, macrophages or lymphocytes. Total cell counts, percent cell viability, and percent differentials from nasal lavage fluid obtained pre-exposure, post-exposure and 24-hours post-exposure are shown in Table B-5 for each subject individually.

No treatment-related trends were seen with protein concentration in nasal lavage supernatant, as shown in Table 6-3. No data are provided in this table for Max CAP exposures since there were only two exposures, and therefore calculation of a mean is not possible for this exposure. The samples were analyzed in duplicate, and the average concentration is presented in the table. The individual data are presented in Table B-6.

Sputum: There were no trends observed in total or differential cell counts in sputum. Individual data are presented in Table B-6 for total and differential cell counts in induced sputum pre-exposure, post-exposure and 24-hours post-exposure. There are seven missing data points, due to insufficient sputum samples obtained for processing. No pollutant-related trends were seen with percent cell viability, neutrophils, macrophages, eosinophils, or lymphocytes. Due to the missing data points and the inter-individual and intra-individual variability in the data, it is not possible to make firm conclusions regarding any trends to increase or decrease in total cell counts or differentials in sputum.

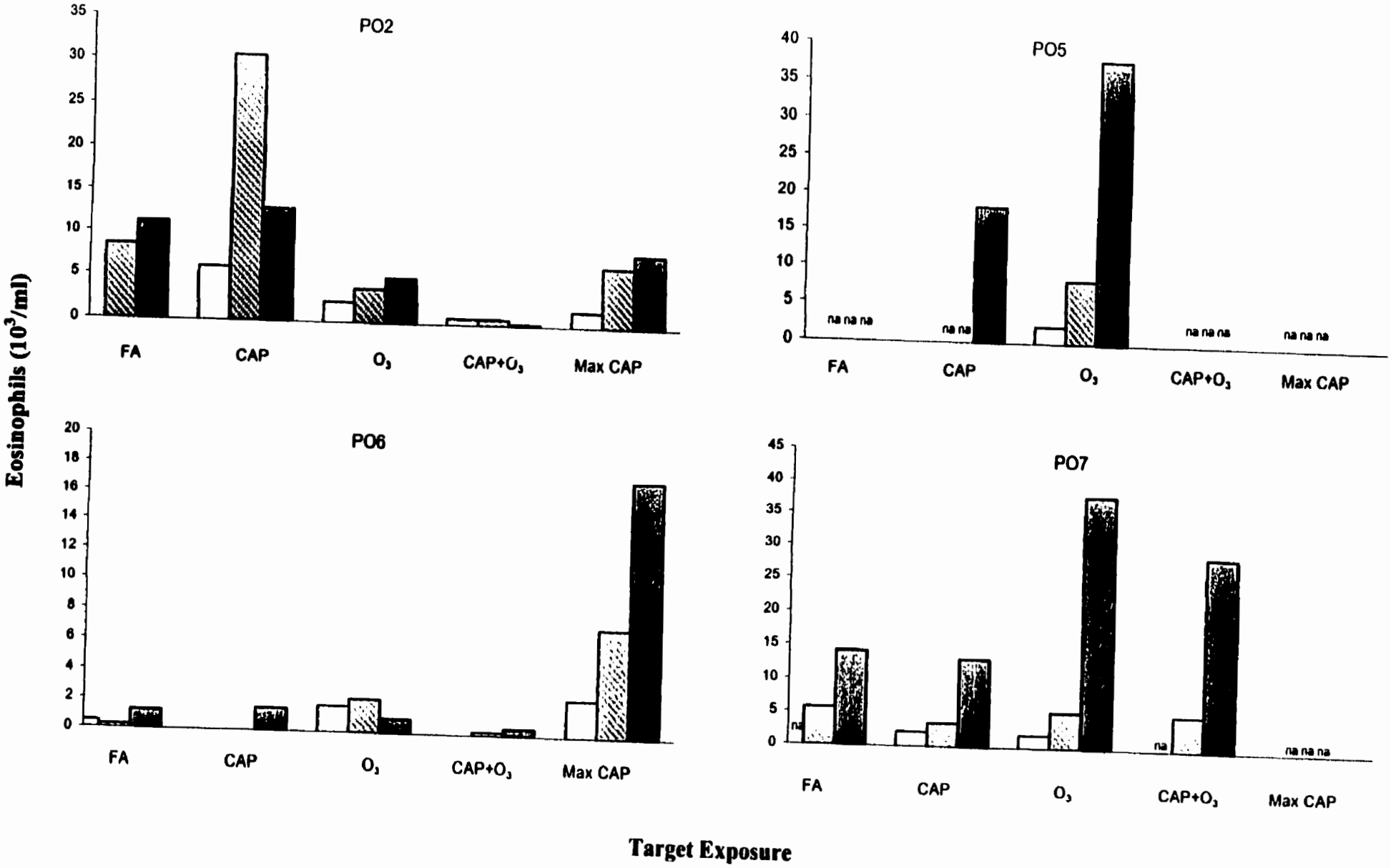

Figure 6-5: Nasal Lavage Eosinophil Counts in Asthmatic Subjects Pre-exposure, Post-exposure and 24-hours Post-exposure

pre-exposure post-exposure 24-hours post-exposure

na = data not available

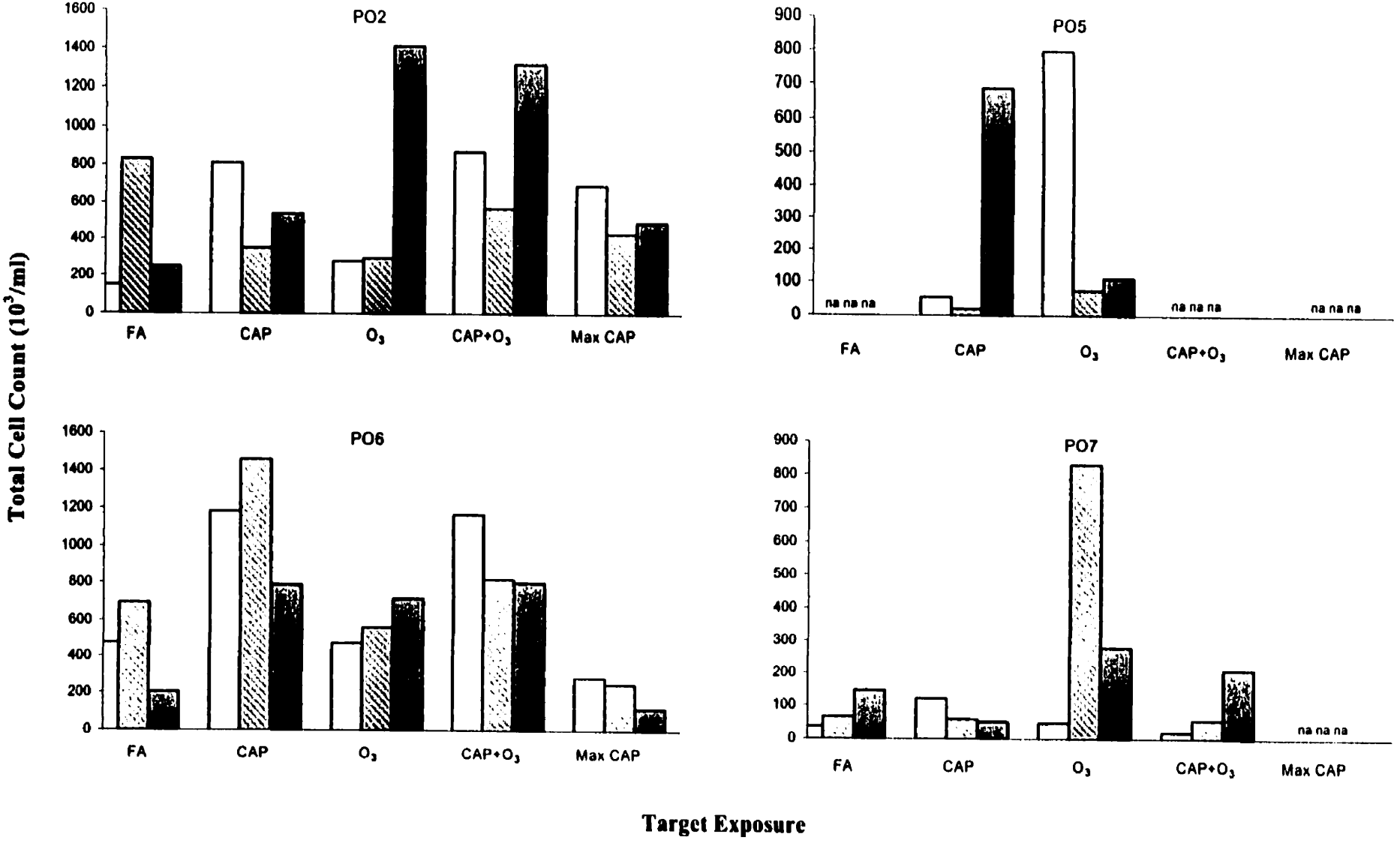

Figure 6-6: Nasal Lavage Total Cell Count in Asthmatic Subjects Pre-exposure, Post-exposure and 24-hours Post-exposure

pre-exposure post-exposure 24-hours post-exposure

na = data not available

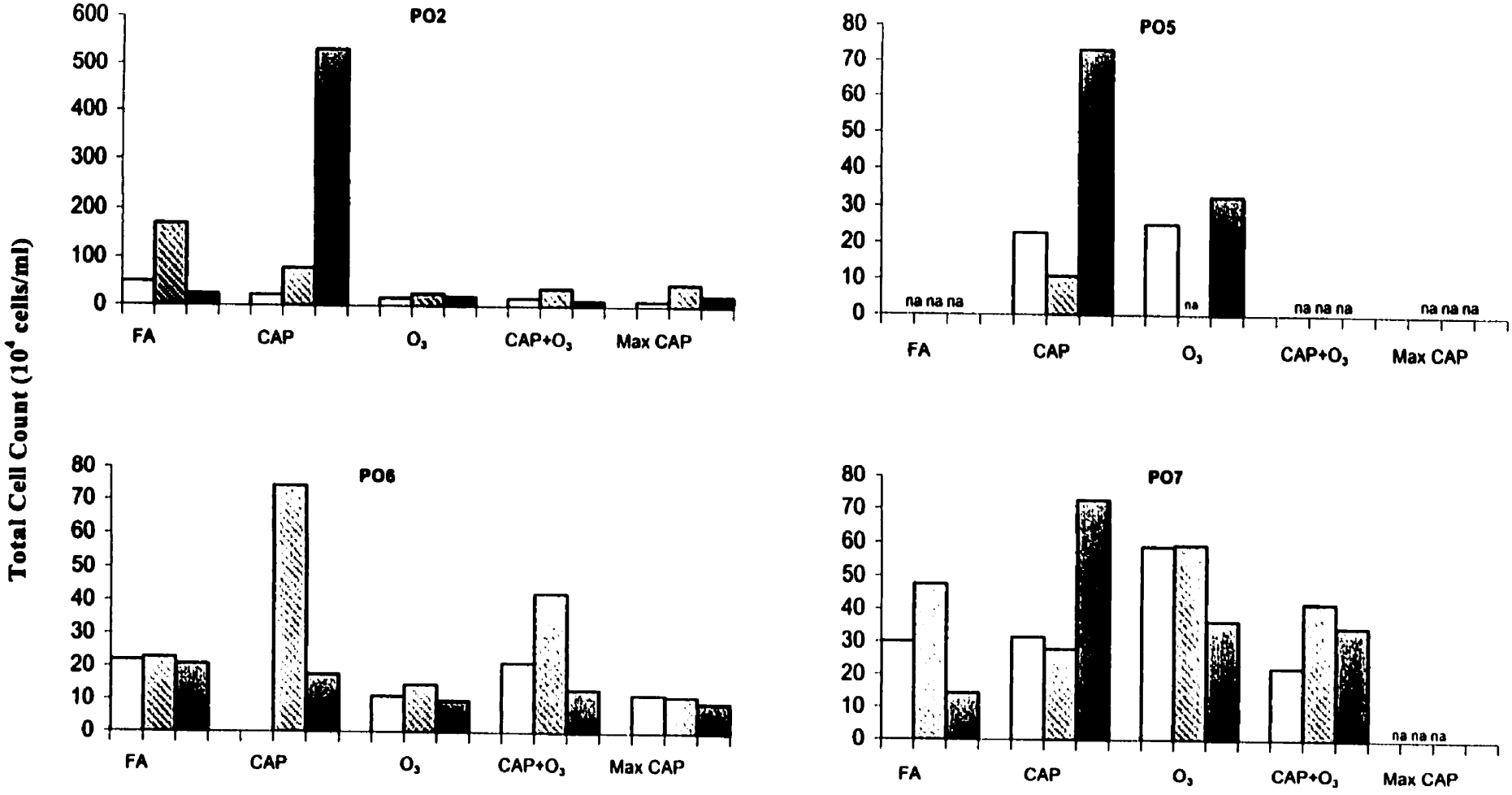

Figure 6-7:Total Cell Counts in Induced Sputum - Asthmatic Subjects Pre-exposure, Post-exposure and 24-hours Post-exposure

pre-exposure post-exposure 24-hours post-exposure

na = data not available

Table 6-3: Mean (± SD) Protein Concentration in Nasal Lavage Fluid and Raw Sputum of Asthmatic Subjects

Target CAP Exposure	Sampling Time	Nasal Lavage Protein Concentration (μg/ml)	Raw Sputum Protein Concentration (μg/ml)
FA	Pre-exposure	928 ± 754	2151 ± 982
	Post-exposure	1345 ± 934	1346 ± 358
	24-hr post-exposure	788 ± 470	1442 ± 403
CAP	Pre-exposure	1198 ± 644	1764 ± 1461
	Post-exposure	1014 ± 508	1549 ± 723
	24-hr post-exposure	919 ± 196	1891 ± 494
O_3	Pre-exposure	855 ± 502	2273 ± 1583
	Post-exposure	1012 ± 512	na
	24-hr post-exposure	1120 ± 536	1557 ± 1114
CAP+O_3	Pre-exposure	1529 ± 1331	2126 ± 1461
	Post-exposure	901 ± 645	1640 ± 1040
	24-hr post-exposure	919 ± 318	1652 ± 899

There did not appear to be any treatment-related effects in protein concentrations analyzed in raw sputum samples (Table 6-3). The average concentration is presented from the samples that were analyzed in triplicate due to the non-homogeneous nature of raw sputum samples.

7.0 DISCUSSION

These preliminary studies were designed as pilot studies using a small number of healthy and mildly asthmatic individuals to examine potential cardiorespiratory effects following exposure to CAP and/or ozone. The aim of the pilot studies was to determine exposure concentrations of CAP for future research with more subjects.

In both Study No. 1 and 2, there were no effects on ECG waveforms to suggest silent ischemia. This lack of cardiotoxicity was anticipated in young healthy or mildly asthmatic individuals exposed to ambient levels of CAP and/or O_3. Similar results were found in a recent study with CAP where there were no effects on ECGs in young, healthy subjects (Ghio *et al.*, 1998). Similarly, no significant ECG effects were found in a controlled human exposure study with 0.3 ppm O_3 (Gong *et al.*, 1998b). This is in contrast to the effects seen in animals exposed to high levels of particles (Godleski *et al.*, 1997; Campen *et al.*, 1996; Nearing *et al.*, 1996; Watkinson *et al.*, 1998a). In these studies, there were effects seen in compromised animals at much higher levels of exposure than used in the current studies. The information obtained from the studies presented in this thesis is preliminary, and the sample size was not sufficient to confirm these results statistically. It is premature to come to any conclusions and therefore, monitoring of ECGs will continue to be very important in future studies, particularly with the elderly, and also with cardiac compromised individuals.

A recent epidemiological study with 90 elderly subjects found a very small increase in heart rate (0.8 beats/min) with an increase in ambient PM_{10} of 100 $\mu g/m^3$ (Pope *et al.*,

1999b). We did not observe any changes in heart rate attributable to particle exposure in the current studies. Controlled human exposure studies with a greater number of subjects are necessary to identify an effect of this small magnitude.

There was a large amount of inter-individual and intra-individual variability with HRV measurements in the current studies, which is consistent with literature for this area. The reproducibility of HRV parameters in normal individuals has not been well studied (Kautzner, 1995). However, some normal values identified from the literature were: 141 ms for SDNN and 27 ms for rMSDD (24-hour recordings); and 1170 ms^2 for LF, 975 ms^2 for HF and 1.5 for LF/HF ratio (5-minute recordings) (Cardiac Task Force, 1996). The size of our data sets in both studies presented in this thesis precluded meaningful statistical analyses. In order to make any firm conclusions regarding effects of pollutant exposures on HRV parameters, it would be necessary to see consistent dose-dependent trends in a small sample size where statistics are not possible. This was not observed in our studies. In general, the data obtained in Study No. 2 were within the range of normal values for healthy individuals.

There was no apparent reduction in HRV, as SDNN, with young healthy or mildly asthmatic individuals exposed to CAP and/or O_3. Similarly, we did not observe a strong trend to change in vagal activity with pollutant exposures compared with FA. Effects of pollutant exposures on HRV parameters may be reflective of sympathetic and vagal activity, and should be examined in a larger sample size in order to determine whether there are statistically significant effects.

In an earlier study, by Bosner and Kleiger (1995) statistically significant differences in HRV parameters between patients who did or did not have increased cardiac mortality were as follows: i) 70 vs. 96 ms for SDNN; ii) 2.25 vs. 4.14 % pNN50; and iii) 27 vs. 32 ms rMSDD. In this study, decreased HRV and decreased vagal activity were associated with increased mortality ($p<0.05$). We did not see a pattern to decreased SDNN or vagal activity in our studies. In fact, there appears to be an increase in vagal activity in some pollutant exposures compared with FA in our studies. However, these results are preliminary, and need to be confirmed in further studies with a larger sample size in order to identify whether there are statistically significant results between groups.

Altered HRV activity has been seen in other studies with air pollutants. In animal studies, vagal activity was increased with CAP exposures in dogs, compared with FA exposures (Lovett *et al.*, 1998). Using a similar study design to the previous study, Lovett *et al.* (1999) found an increase in LF, HF and LF/HF with CAP exposure in mongrel dogs compared with FA exposures. In a controlled clinical study with SO_2, healthy individuals had decreases in total power, HF and LF, whereas asthmatics had increases in total power, HF and LF with SO_2 vs. FA exposures (Tunnecliff *et al.*, 1999). Recent data from another laboratory conducting CAP exposures with humans have found changes in HRV 24-hours post-exposure (personal communication, A. Ghio, US EPA, North Carolina) but no significant changes in HRV during exposure. Based on these unpublished results, future studies should include analysis of HRV 24-hours post-exposure.

The HRV monitoring conducted for the US EPA studies with CAP were done while the subjects were resting in a dark room (personal communication, A. Ghio, US EPA, North

Carolina). This is important to minimize noise on the ECG recording and maximize the data recovered on the ECG for subsequent HRV analysis. This is particularly important for cardiac compromised individuals where the likelihood of ectopic beats is more common than in young, healthy individuals. It is also an excellent method of ensuring that the subjects are all performing the same activity since standing/sitting, mental stress, and altered breathing will affect the HRV parameters. Additionally, as mentioned above, this minimizes the potential for noise on the recording. For these reasons, it would be prudent for future studies to monitor HRV pre-exposure, post-exposure and 24-hours post-exposure be recorded during resting of the subject for 10 to 15 minutes, in order to minimize noise during recording.

Inflammation of lung tissue following exposure to pollutants may be accompanied by increased blood coagulant activity (Seaton *et al.*, 1995), such as plasma fibrinogen and clotting factor VII antigen. Blood coagulant activity is increased in the body in order to wall off areas of inflammation. In Study No. 1, there appeared to be a trend to increased plasma fibrinogen levels following the high CAP exposure exercise period in healthy individuals. Following exercise, there was a mean increase of approximately 10% with high CAP vs. only 2% with FA exposure. The mean values were 319 mg/dl with high CAP and 279 mg/dl with FA post-exercise. It is recognized that exercise causes an acute increase in fibrinogen (Montgomery *et al.*, 1996), but this was not a confounder in our study since the values post-exercise following particle exposures were compared to values post-exercise at the control exposure. In Study No. 2, there was no strong trend to increased fibrinogen levels following exposure to CAP and/or O_3. Due to subject attrition

and the presence of blood clots in several plasma samples, there was too much variability in the remaining samples to make any conclusions.

Fibrinogen levels have been elevated in toxicological studies with rats exposed to oil fly ash particles (Gardner and Costa, 1998; Kodavanti *et al.*, 1999). It is also of interest to note that plasma fibrinogen levels are increased during exacerbation in COPD patients (Seemungal *et al.*, 1999), who may be a susceptible population for air pollution episodes because of their compromised lung function. Fibrinogen is associated with atherosclerosis and increased risk of ischemic heart disease (Sweetnam *et al.*, 1996; Levenson *et al.*, 1995), which may be due to increased blood viscosity (Sweetnam *et al.*, 1996). Atherosclerosis may be due to increased damage of artery wall endothelium or smooth muscle cells, resulting in inflammation (Levenson *et al.*, 1995). In studies of heart disease patients, mean levels of fibrinogen of 7 to 34 mg/dl above controls were a long-term predictor of cardiovascular disease or mortality (Tracy *et al.*, 1995; Sweetnam *et al.*, 1996). The increases seen in Study No. 1 were in this range or higher, although it must be noted that these studies measured acute rises in fibrinogen, rather than increased chronic levels. The finding of a trend to increased plasma fibrinogen levels compared to the control is of considerable interest and should be examined further in a larger sample size.

Since fibrinogen levels in the body are controlled by homeostatic mechanisms, it would be of interest to identify whether the increase in plasma fibrinogen levels is reflected in an increase in sputum fibrinogen levels. An increase in sputum fibrinogen would be reflective of increased epithelial permeability following tissue damage (Pizzchini *et al.*,

1997). No such effect was seen with total protein concentrations in respiratory tract lining fluid in our study; but there could be an increase in one protein that is not reflected by the test for total protein. Further to this, the cytokine IL-6 levels should be examined in sputum and plasma, since IL-6 is part of the biofeedback mechanism for fibrinogen. Activated macrophages release IL-6, which then circulates and stimulates the liver to release fibrinogen, which would result in increased plasma fibrinogen levels. This information then would substantiate the biological plausibility of an increase in plasma fibrinogen resulting from an inflammatory response to CAP.

We did not see any trends to increase in plasma clotting factor VII antigen in Study No. 1 and therefore elected to exclude this parameter in Study No. 2. This is consistent with the results of another recent study that found increased fibrinogen levels but not factor VII following air pollution episodes (Pekkanen *et al.*, 1999).

The nominal levels of CAP in this study were not associated with a strong cellular inflammatory response in the respiratory tract lining fluid of either the healthy or asthmatic volunteers. Neutrophils, eosinophils and macrophages are recruited into tissue from the blood during an inflammatory response for phagocytosis of foreign agents. An increase in these cells in nasal fluid or induced sputum is indicative of an inflammatory response. In Study No. 1, there was an apparent increase in macrophages in the nasal lavage fluid samples of healthy subjects with CAP vs. FA exposures, but it was not possible to interpret the biological significance of this increase. This is because there are generally a small absolute number of macrophages in nasal lavage fluid, and the increase in the small number of macrophages present in nasal lavage fluid may be due to chance in

this small sample size. Normally, neutrophils are the major white blood cell type in nasal lavage fluid, with few macrophages present. It is possible that the increase in macrophages seen in the nasal lavage fluid was due to contamination from red blood cells, as blood clots were present in some samples from subjects. The increase in macrophages in nasal lavage fluid therefore needs to be examined in a larger sample size, where there are no blood clots in the samples to confound the results. Only macrophages present in nasal lavage fluid are representative of tissue inflammation, since white blood cells present in blood clots resulting from vigorous nose blowing are not indicative of a tissue inflammatory response. Blood clots in nasal lavage fluid did not appear to be more frequent in CAP vs. FA exposures, or post-exposure vs. pre-exposure.

In Study No. 1, there was an increase in neutrophils in nasal lavage fluid of healthy subjects in 7/10 samples post-exposure and 8/9 samples 24-hours post-exposure to CAP but only in one subject following FA. No dose-response was evident with target CAP mass concentrations. The increase in nasal neutrophils was not seen in asthmatics in the second study, although there was an increase in the percent eosinophils that was more pronounced for CAP than FA. These results suggest there may be a mild inflammatory response in the nose of both healthy and asthmatic individuals following CAP exposure. Further study is required with more subjects to identify whether this trend to increased inflammatory cells in nasal lavage fluid is statistically significant in a larger sample size. This is important since, although some studies with particles have found increased inflammation suggested by increases in neutrophils (Koren *et al.*, 1990; McBride *et al.*, 1994), other studies have found variable results in cell counts (Peden *et al.*, 1995; Torres *et al.*, 1997). Although no consistent dose-response trends for cellular inflammation in

the nose were evident in our studies, it is possible that this is due to inter-individual or intra-individual variability with a small sample size. This can be resolved in a larger sample size where a statistical evaluation of the data is possible.

There was no dose-response trend observed that is indicative of an inflammatory response in induced sputum samples of either healthy or asthmatic subjects. This may be a result of the relatively low exposure levels with healthy and asthmatic individuals in these preliminary safety studies, or it may be a result of the small number of samples, since it was not always possible to obtain a sufficient sample of sputum for processing. Our initial study did not include a pre-exposure measure of sputum and that hampered interpretation of the data due to the intra-individual variability in this parameter on different days. In the second study, although there was a pre-exposure collection of sputum samples, it was not always possible to obtain a good sample for processing pre- and post-exposure, and therefore we did not obtain a sufficient number of data points to make firm conclusions regarding a cellular inflammatory response. However, it is noted that no significant inflammation was observed with bronchoscopy in studies with a larger sample size, where 24 healthy subjects were exposed to 50 to 500 $\mu g/m^3$ CAP (Ghio and Devlin, 1999).

It is considered possible in the scientific community that health effects associated with increases in $PM_{2.5}$ may be due to an unknown chemical constituent or biological entity, and not on mass concentration (McLellan and Miller, 1997), but this was not assessed in the present study. There currently are many studies being conducted with the inorganic constituents of particles to identify whether the adverse health effects observed in

epidemiological studies are a result of individual chemical constituents rather than PM mass. Study of the chemical composition of $PM_{2.5}$ will aid in the determination of the biological mechanism of effect of PM toxicity. For instance, metals on particles such as vanadium that is known to have cardiotoxic effects, may be responsible for some cardiotoxicity of ambient particulate matter. For the current studies, it was not possible to analyze the metal content of the particles from the filters collected during exposure since there was insufficient mass on the filters. It is suggested that the metal content of the filters from exposures be analyzed in future studies to identify whether there are dose-response relationships associated with some common inorganic constituents of ambient particles.

The pilot safety studies summarized in this thesis provide data regarding several variables that may be associated with cardiorespiratory illness. The purpose of the studies was to serve as a basis for future studies in this area. The studies were not designed to provide statistically significant results regarding potential cardiorespiratory health effects. There is a lot of intra- and inter-individual variability associated with each of the indicators used in these studies. This is evident by the large standard deviation values for the biological parameters monitored, as well as the CAP measurements, as shown in the tables presented in the results sections. Although the studies were designed to minimize confounding due to inter-individual variability in this small sample size, it is noted that intra-individual variability was also evident in the parameters measured.

There were not enough subjects in our pilot studies to evaluate whether the results are statistically significant. In order to have sufficient statistical power to analyze the results

from studies such as these, it is necessary to have more subjects in the study. The number of subjects required is determined based on the variability in the parameters measured. As indicated in the text, Table A-8, Table A-9 and tables in Appendices C and D, there is a lot of inter-individual and intra-individual variability in the environmental and biological parameters assessed in this study. Using the following equation for statistical power (Snedecor, 1962) and the fibrinogen data for Study No. 1, it is estimated that a sample size of 30 will be required in future studies to see a change between groups following exposure compared with pre-exposure. The equation used for the calculation of the sample size was based on the data obtained in our studies post-exercise compared with pre-exposure:

$$\delta^2 = \frac{(S_D \times t_{05})^2}{n^2}$$

where: δ is the difference we want to observe (e.g., the difference of the pooled mean change for the mid and high CAP exposures minus the difference in fibrinogen post-exercise - pre-exposure during the FA exposure, or 9 mg/dl); S_D is the standard deviation of the differences of fibrinogen post-exercise compared to pre-exposure during the high CAP and FA exposures (24.47); and t_{05} is 2.045 for 29 degrees of freedom.

In the current studies, the CAP mass concentrations selected for the studies were those suggested by epidemiological studies to cause increased morbidity and mortality, but not so high that they would be expected to cause serious health concerns in our subjects. Due to ethical reasons, it is important to select concentrations of pollutants that are expected to cause a mild and transient response. Based on the results of the present studies, there

does not appear to be a dose-response relationship with CAP based on mass concentration at the levels studied in young healthy or mildly asthmatic individuals.

In outdoor air, ambient gaseous pollutants coexist with particles and there is a potential for synergistic or additive effects. In a recent animal study it was shown that inhalation of urban particulate matter potentiated ozone effects on lung damage (Bouthellier *et al.*, 1998). In our second study, we did not find additive or synergistic effects with combined exposure to CAP and ozone at levels up to and above those found on a smoggy day in Toronto. Similarly, we did not identify effects in asthmatics following exposure to 80 ppb ozone alone using the parameters examined in this study. Although respiratory effects have been seen at this concentration in short-term studies (two to seven hours) (Koenig, 1999), most controlled human exposure studies with ozone use more than 200 ppb ozone (Aris *et al.*, 1995; Frampton *et al.*, 1997; Gong *et al.*, 1998b). Our data suggest that exposure to up to 130 $\mu g/m^3$ CAP plus 80 ppb ozone does not cause adverse health effects in the variables measured, although it is possible that there may be effects at higher exposure levels or in more susceptible individuals.

8.0 CONCLUSION

In conclusion, we were not able to demonstrate that levels up to 130 $\mu g/m^3$ CAP with or without 80 ppb ozone had cardiorespiratory effects in young, healthy or asthmatic subjects using the parameters monitored in these studies. These levels are similar to or above smoggy day levels in Toronto, and it is generally accepted that adverse cardiorespiratory effects seen in epidemiological studies at ambient levels are reflective of effects on sensitive members of the population, rather than on young, healthy people. This has been further observed in animal toxicological studies in which effects were greater in compromised animals, compared with healthy animals. These pilot studies were conducted to determine the safety of CAP exposures in larger clinical studies. They were not expected to provide statistically significant effects, rather to generate enough data to provide information for future studies with more subjects, and with more susceptible subjects.

These preliminary studies identified that levels of up to 130 $\mu g/m^3$ CAP can be used in further research to identify biological mechanisms of toxicity of ambient fine particles. The addition of gaseous pollutants to the CAP exposures is also important to further examine potential synergistic or additive effects. Our findings suggest that further research is required on $PM_{2.5}$ at higher exposure levels and as well, there is a need to examine effects in more susceptible groups of the population in order to elucidate the biological mechanism of action and determine dose-response relationships for $PM_{2.5}$.

9.0 FUTURE DIRECTIONS

Further research is required to elucidate the biological mechanisms of toxicity for ambient $PM_{2.5}$ at exposure levels experienced by the general population. Additionally, dose-response relationships need to be identified in order for regulations to be established for $PM_{2.5}$ using a cost-benefit analysis. Regulators consider the no-observed-adverse-effect level (NOAEL) or the lowest-observed-adverse-effect level (LOAEL) of a pollutant in ambient air in order to provide air quality objectives designed to protect the general population (CCME, 1999a). The current reference levels for $PM_{2.5}$ and PM_{10} in Canada and the air quality objectives in the US are based largely on epidemiological studies. Further studies are required to determine a LOAEL or NOAEL from controlled human exposure studies or animal toxicological studies to provide insight on the biological mechanisms associated with the adverse health effects observed in the epidemiological studies.

As demonstrated in the current preliminary studies, exposure to $PM_{2.5}$ at ambient levels up to and above those found on a smoggy day in Toronto does not appear to result in cardiorespiratory effects in a small number of young, healthy or mildly asthmatic individuals using the parameters monitored. It is possible that these levels may have effects in more susceptible individuals or that subtle effects may be more evident in a larger sample size. Some recommendations for further research are outlined below:

- Test a greater number of subjects in order to expand on the findings from the preliminary studies, ensuring an adequate number of people to obtain statistically significant results;

- Use more susceptible individuals with respiratory disease, such as asthmatics that tend to react to air pollutants (e.g., ozone responders). Additionally, people with abnormal HRV, such as the elderly, diabetics, and cardiac compromised individuals who have had a myocardial infarction (Klieger *et al.*, 1995) could be used as susceptible populations;

- Use higher $PM_{2.5}$ concentrations to simulate pollution episodes in cities such as Los Angeles or Mexico City in order to observe health effects, but still maintain realistic levels of exposure for evaluation of biological mechanisms; and,

- Expose subjects to combinations of multiple gaseous pollutants (e.g., $\geq$2 different pollutants) and $PM_{2.5}$, including O_3, NO_X, SO_2, and CO based on the results of epidemiological studies using multipollutant models.

In addition to recommendations for the types of subjects and exposures in future studies listed above, there are some suggestions for improvement of tests conducted during exposures:

- HRV analyses should be obtained during resting of each subject for 15 minutes pre-exposure, post-exposure and 24-hours post-exposure. This will ensure that the measurements are taken for the same activity, and will minimize noise on recordings. HRV should also be assessed during exposure, exercise, and recovery following exercise;

- Additional plasma samples should be obtained for duplicate fibrinogen analyses to minimize the potential for lost samples due to blood clots or storage for future analyses and to study variability of the method;

- Cytokine IL-6 levels should be examined in sputum and plasma, since IL-6 is part of the biofeedback mechanism for fibrinogen. Activated macrophages release IL-6, which then circulates and stimulates the liver to release fibrinogen, which would result in increased plasma fibrinogen levels. This information then would substantiate the biological plausibility of an increase in plasma fibrinogen resulting from an inflammatory response to CAP; and

- The chemical constituents of $PM_{2.5}$ samples during exposures should be analyzed for metals, sulfates, and organics in order to determine whether health effects are associated with chemical constituents of particles. The valence state of the metals should be determined since not all valence states will have biological activity.

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Table A-1: Mean (±SE) PM and Gases in HEF During Exposure for Healthy Subjects

Subject/Sample	FA	low CAP	mid CAP	high CAP
$PM_{2.5}$ gravimetric ($\mu g/m^3$)				
PS2	5.96	22.72	23.73	89.36
PS3	0*	na	44.8	91.63
PS5	0*	33.58	na	63.63
PS7	4.07	38.18	90.1	123.7
Ozone (ppb)				
PS2	0.10 ± 0.00	0.64 ± 0.05	4.94 ± 0.28	6.43 ± 0.37
PS3	na	19.55 ± 0.36	4.73 ± 0.35	10.34 ± 0.26
PS5	22.29 ± 0.14	5.07 ± 0.48	25.88 ± 0.47	4.11 ± 0.38
PS7	5.49 ± 0.35	18.4 ± 0.46	6.63 ± 0.36	7.14 ± 0.40
Carbon monoxide (ppm)				
PS2	0.51 ± 0.01	0.34 ± 0.01	0.45 ± 0.01	0.60 ± 0.02
PS3	na	0.07 ± 0.01	0.31 ± 0.02	0.55 ± 0.01
PS5	na	0.26 ± 0.01	0.01 ± 0.01	na
PS7	0.20 ± 0.01	0.22 ± 0.01	0.44 ± 0.02	0.85 ± 0.04
Nitrogen dioxide (ppb)				
PS2	14.67 ± 0.13	15.83 ± 0.14	23.37 ± 0.12	21.09 ± 0.25
PS3	na	7.35 ± 0.10	15.01 ± 0.18	24.42 ± 0.22
PS5	na	29.71 ± 0.13	9.82 ± 0.14	22.16 ± 0.42
PS7	20.00 ± 0.17	16.52 ± 0.20	27.17 ± 0.27	33.22 ± 0.53
Nitric oxide (ppb)				
PS2	14.04 ± 0.11	20.76 ± 0.60	16.69 ± 0.25	27.36 ± 0.60
PS3	na	4.99 ± 0.06	9.44 ± 0.19	9.86 ± 0.34
PS5	0.25 ± 0.01	21.76 ± 0.55	4.43 ± 0.14	14.56 ± 0.69
PS7	13.00 ± 0.33	6.17 ± 0.10	36.75 ± 0.95	76.68 ± 1.5
Sulfur dioxide (ppb)				
PS2	2.94 ± 0.13	1.04 ± 0.07	0.81 ± 0.02	0.24 ± 0.11
PS3	na	0.45 ± 0.02	1.18 ± 0.08	3.31 ± 0.06
PS5	11.23 ± 0.15	0.20 ± 0.06	0.51 ± 0.01	1.91 ± 0.20
PS7	2.52 ± 0.10	na	7.24 ± 0.59	2.71 ± 0.06

Note: no SE for PM since only one data point available

na = data not available

* Note: there was measurement variability at low masses, resulting in negative values.

These values are reported as 0 $\mu g/m^3$

Table A-2: Plasma Fibrinogen and Factor VII Concentrations in Healthy Subjects - Individual Data

Subject/Sample	Fibrinogen (mg/dl)				Factor VII (%)			
	FA	Low CAP	Mid CAP	High CAP	FA	Low CAP	Mid CAP	High CAP
PS2								
pre-exposure	340	348	313	350	92	97.7	92.5	92.3
post-exposure	348	365	324	331	96.5	102.1	97.2	98.6
post-exercise	358	363	303	406	105.4	99	100.8	98.4
24-hr post-exposure	336	410	311	391	91.1	92.8	92.3	100.5
PS3								
pre-exposure	295	312	263	368	98	94.8	99.4	103
post-exposure	336	na	257	420	105.3	na	102.2	100.9
post-exercise	313	313	274	439	96	99.1	115.4	98.3
24-hr post-exposure	312	na	294	na	104	na	103	na
PS5								
pre-exposure	229	236	216	197	96.3	98.7	92.5	99.7
post-exposure	240	233	216	198	98.9	98.7	99.6	109.1
post-exercise	226	225	206	206	108.1	99	114.4	101.1
24-hr post-exposure	244	225	219	198	99.2	109.3	100.5	99.6
PS7								
pre-exposure	222	224	221	230	89.5	94.2	92.8	104
post-exposure	221	219	236	233	92.5	95.8	95.5	107.8
post-exercise	219	215	232	226	88.7	96.6	93	95.5
24-hr post-exposure	223	229	234	223	98.5	95.4	105.2	104.7

na = it was not possible to obtain a blood sample at this time

Where: FA = filtered air; Low CAP = target of 20 $\mu g/m^3$; Mid CAP = target of 40 $\mu g/m^3$; and High CAP = target of 60 $\mu g/m^3$

Table A-3: Heart Rate Variability Parameters in Healthy Subjects
Mean Values for Exposure Period (120 min Duration)

Target Exposure	LF power $(beats/min)^2$	HF power $(beats/min)^2$	LF/HF ratio (unitless)	Ave Heart Rate (beats/min)	SDNN (beats/min)
PS2					
FA	5.56	3.74	1.64	53	4.30
Low CAP	8.37	6.69	1.47	56	5.73
Mid Cap	11.46	6.83	1.74	57	6.24
High CAP	10.59	7.72	1.69	61	6.51
PS3					
FA	2.35	1.67	1.51	77	3.10
Low CAP	2.64	5.37	0.53	76	4.75
Mid CAP	2.65	1.40	1.78	83	3.10
High CAP	3.75	1.70	2.07	86	3.46
PS5					
FA	8.96	3.99	2.10	58	4.88
Low CAP	6.15	3.34	1.50	57	3.89
Mid CAP	5.92	4.55	1.58	55	4.29
High CAP	6.09	4.74	1.27	54	4.41
PS7					
FA	16.65	6.86	2.65	73	6.33
Low CAP	13.62	8.30	1.64	65	5.98
Mid CAP	18.46	7.61	2.36	69	6.81
High CAP	14.77	5.12	3.15	76	5.77

Table A-4: Heart Rate Variability Parameters in Healthy Subjects
Mean Values for Exercise Period (30 min Duration)

Target Exposure	LF power $(beats/min)^2$	HF power $(beats/min)^2$	LF/HF ratio (unitless)	Ave Heart Rate (beats/min)	SDNN (beats/min)
PS2					
FA	2.32	0.43	5.76	123	3.05
Low CAP	2.88	0.79	4.39	126	3.63
Mid Cap	6.01	1.13	7.73	123	4.62
High CAP	3.17	0.85	5.56	121	3.45
PS3					
FA	1.67	0.27	4.84	125	2.40
Low CAP	2.47	4.42	0.61	128	7.04
Mid CAP	1.27	0.54	2.33	130	2.87
High CAP	1.24	0.33	3.35	125	2.65
PS5					
FA	3.13	1.01	2.77	124	3.06
Low CAP	3.95	1.23	3.23	127	4.28
Mid CAP	4.67	1.20	2.64	125	3.69
High CAP	3.79	1.53	2.24	126	4.02
PS7					
FA	7.53	3.39	2.48	127	4.26
Low CAP	8.14	4.10	2.02	118	4.49
Mid CAP	5.50	3.60	2.06	124	4.48
High CAP	4.83	7.09	0.95	124	6.29

Table A-5: Nasal Lavage Total Cell Count and Percent Differentials in Healthy Subjects - Individual Data

Target CAP	Sampling Time	Cell Viability (%)	Total cell count (10^3 cells/ml)	Epithelial Cells (%)	Neutrophils (%)	Macrophages (%)	Eosinophils (%)	Lymphocytes (%)
PS2								
FA	pre-exposure	80	247	57	42	0.8	0.5	0.0
	post-exposure	83	184	66	31	1.4	0.0	1.0
	24 hr post	80	109	63	34	0.8	1.6	0.0
Low CAP	pre-exposure	77	87	70	27	0.7	0.3	2.4
	post-exposure	76	404	39	54	2.8	1.0	3.5
	24 hr post	73	147	45	51	1.8	0.3	2.0
Mid CAP	pre-exposure	66	1484	49	49	0.5	0.3	0.8
	post-exposure	83	180	33	65	1.5	0.3	0.8
	24 hr post	84	216	43	53	1.8	0.3	2.0
High CAP	pre-exposure	71	340	45	53	0.3	1.0	0.8
	post-exposure	60	584	56	42	1.0	0.8	0.5
	24 hr post	63	216	44	55	1.0	0.0	0.8
PS3								
FA	pre-exposure	82	187	33	60	0.0	4.8	2.1
	post-exposure	46	456	32	58	1.3	7.8	0.8
	24 hr post	57	367	62	35	0.8	1.8	0.3
Low CAP	pre-exposure	64	536	na	na	na	na	na
	post-exposure	89	91	27	68	1.2	2.5	1.9
	24 hr post	60	129	19	69	0.0	11.6	0.0
Mid CAP	pre-exposure	49	211	61	25	2.0	9.0	3.0
	post-exposure	80	120	48	45	1.1	4.0	2.3
	24 hr post	68	775	60	34	1.5	4.0	0.3
High CAP	pre-exposure	75	420	27	71	0.3	1.3	0.8
	post-exposure	38	460	44	53	0.0	2.0	0.8
	24 hr post	66	780	36	60	1.0	2.8	0.3
PS5								
FA	pre-exposure	63	158	86	14	0.0	0.7	0.0
	post-exposure	66	264	86	8	1.0	0.3	5.0
	24 hr post	41	189	93	6	0.5	0.0	1.3
Low CAP	pre-exposure	51	224	92	6	0.0	0.0	2.5
	post-exposure	48	3577	84	14	0.3	0.3	1.5
	24 hr post	48	311	na	na	na	na	na
Mid CAP	pre-exposure	60	631	85	14	0.3	0.0	1.5
	post-exposure	45	551	88	12	0.3	0.0	0.3
	24 hr post	58	684	74	20	0.5	0.3	1.0
High CAP	pre-exposure	25	2411	na	na	na	na	na
	post-exposure	43	831	96	3	0.0	0.0	0.8
	24 hr post	35	1180	na	na	na	na	na
PS7								
FA	pre-exposure	44	695	88	11	0.8	0.0	0.5
	post-exposure	68	615	67	31	0.5	0.5	1.3
	24 hr post	55	1100	64	34	0.8	0.0	1.0
Low CAP	pre-exposure	57	1000	88	11	0.3	0.8	0.5
	post-exposure	70	724	54	43	0.3	0.3	2.5
	24 hr post	47	1164	60	38	1.5	0.0	0.5
Mid CAP	pre-exposure	35	1531	85	14	0.3	0.3	0.8
	post-exposure	55	1191	34	62	0.0	0.5	0.0
	24 hr post	47	1555	61	36	1.8	0.0	1.3
High CAP	pre-exposure	44	960	68	28	0.3	1.3	2.8
	post-exposure	67	940	51	48	0.8	0.3	0.5
	24 hr post	40	700	22	72	1.5	3.0	2.5

Table A-6: Protein Concentration in Nasal Lavage Fluid and Raw Sputum in Healthy Subjects - Individual Data

Target CAP Exposure	Sampling Time	Nasal Lavage Protein Concentration (µg/ml)	Raw Sputum Protein Concentration (µg/ml)
PS2			
FA	pre-exposure	454	-
	post-exposure	399	1211
	24-hr post-exposure	694	1593
Low CAP	pre-exposure	567	-
	post-exposure	682	1225
	24-hr post-exposure	799	1520
Mid CAP	pre-exposure	657	-
	post-exposure	593	1333
	24-hr post-exposure	607	1355
High CAP	pre-exposure	416	-
	post-exposure	389	1793
	24-hr post-exposure	395	2304
PS3			
FA	pre-exposure	333	-
	post-exposure	524	1344
	24-hr post-exposure	386	1155
Low CAP	pre-exposure	722	-
	post-exposure	530	1542
	24-hr post-exposure	791	na
Mid CAP	pre-exposure	561	-
	post-exposure	403	1185
	24-hr post-exposure	701	na
High CAP	pre-exposure	260	-
	post-exposure	494	na
	24-hr post-exposure	462	na
PS5			
FA	pre-exposure	618	-
	post-exposure	548	2356
	24-hr post-exposure	490	3345
Low CAP	pre-exposure	407	-
	post-exposure	495	3988
	24-hr post-exposure	797	4880
Mid CAP	pre-exposure	759	-
	post-exposure	696	5241
	24-hr post-exposure	929	3440
High CAP	pre-exposure	979	-
	post-exposure	565	2159
	24-hr post-exposure	783	2634
PS7			
FA	pre-exposure	767	-
	post-exposure	905	2577
	24-hr post-exposure	1120	2851
Low CAP	pre-exposure	639	-
	post-exposure	845	2154
	24-hr post-exposure	938	2559
Mid CAP	pre-exposure	637	-
	post-exposure	915	3699
	24-hr post-exposure	690	3618
High CAP	pre-exposure	614	-
	post-exposure	997	2308
	24-hr post-exposure	1061	2746

Note: no pre-exposure sputum samples taken in Study No. 1
na = data not available due to insufficient sample

Table A-7: Sputum Total Cell Counts and Percent Differentials in Healthy Subjects - Individual Data

Subject	Target CAP Exposure	Cell Viability (%) post	Cell Viability (%) 24-hour	Total cell count (10^3 cells/ml) post	Total cell count (10^3 cells/ml) 24-hour	Neutrophils (%) post	Neutrophils (%) 24-hour	Macrophages (%) post	Macrophages (%) 24-hour	Eosinophils (%) post	Eosinophils (%) 24-hour	Lymphocytes (%) post	Lymphocytes (%) 24-hour
PS2	FA	76	81	313	564	75.00	66.00	20.25	29.75	3.00	1.75	1.75	2.50
	Low CAP	54	61	831	580	20.50	30.75	75.25	63.75	0.00	0.25	3.25	4.75
	Mid CAP	54	67	476	675	43.50	50.25	51.75	44.00	1.00	1.00	3.75	4.75
	High CAP	63	61	456	411	46.25	54.75	51.75	40.00	0.75	1.50	1.25	3.75
PS3	FA	64	69	404	187	9.00	20.75	87.50	72.50	0.25	0.75	3.25	5.25
	Low CAP	60	na	227	na	7.00	na	89.50	na	0.25	na	2.50	na
	Mid CAP	56	na	196	na	na	na	na	na	na	na	na	na
	High CAP	na	na	na	na	na	na	na	na	na	na	na	na
PS5	FA	72	74	1100	536	81.00	70.75	15.25	23.50	0.25	0.00	3.50	5.75
	Low CAP	73	70	620	8206	62.75	84.75	34.50	13.00	0.50	0.25	2.25	2.00
	Mid CAP	58	63	1220	1300	44.25	69.25	51.75	26.25	0.25	0.25	3.75	4.25
	High CAP	55	na	855	na	24.75	na	69.00	na	0.00	na	6.00	na
PS7	FA	72	75	811	951	14.50	27.75	82.50	66.50	0.00	1.00	1.50	4.00
	Low CAP	70	76	1015	1175	20.00	43.25	74.00	52.25	0.00	0.25	5.50	4.00
	Mid CAP	68	77	744	904	22.75	37.25	73.50	57.50	0.00	0.00	3.75	5.25
	High CAP	77	76	984	544	23.50	40.25	70.25	53.00	0.00	1.25	5.00	4.50

Sampling Times are: post = post-exposure and 24-hour = 24 hours post-exposure

Table A-8: Intraindividual Variability for Subject PS2

Parameter	Mean ± SD	Coefficient of Variation (%)
Blood		
fibrinogen (mg/dl)	338 ± 17	5
factor VII (%)	94 ± 3	3
Nasal Lavage		
cell viability (%)	74 ± 6.2	8
total cell count (10^3 cells/ml)	539 ± 639	118
epithelial cells (%)	55 ± 11	20
neutrophils (%)	43 ± 11	27
macrophages (%)	0.5 ± 0.2	41
eosinophils (%)	0.5 ± 0.3	64
lymphocytes (%)	1.0 ± 1.0	103
protein concentration (µg/ml)	524 ± 110	21

Note: Variability shown as mean ± SD for pre-exposure measurements
Note: no values possible for sputum as no pre-exposure samples taken in Study No. 1

Table A-9: Methodological Variability in Plasma Fibrinogen

Subject/Sample	Fibrinogen (mg/dl)
Female	
Sample 1	1.93
Sample 2	1.86
Sample 3	1.86
Sample 4	1.86
Sample 5	1.86
Mean	1.87
Standard Deviation	0.03
Coefficient of Variation (%)	1.6
Male	
Sample 1	1.56
Sample 2	1.38
Sample 3	1.38
Sample 4	1.67
Sample 5	1.50
Mean	1.50
Standard Deviation	0.12
Coefficient of Variation (%)	8.0

Note: methodology from Study No. 2
Note: To estimate methodological variability, two subjects had five blood samples taken at the same time.

Table B-1: Mean (±SE) PM and Gases in HEF During Exposure for Individual Asthmatic Subjects

Subject/Sample	FA	CAP	O_3	CAP+O_3	Max CAP
$PM_{2.5}$ gravimetric ($\mu g/m^3$)					
PO2	2.5	55.3	4.5	47.8	106.1
PO5	na	48.5	7.1	na	na
PO6	0.5	31.8	3.0	122.7	130.1
PO7	0.5	51.3	1.0	72.2	na
Ozone (ppb)					
PO2	2.39 ± 0.05	0.40 ± 0.01	75.55 ± 1.02	82.96 ± 0.51	9.30 ± 0.37
PO5	na	3.15 ± 0.04	86.01 ± 0.44	na	na
PO6	20.05 ± 0.18	2.17 ± 0.03	85.34 ± 0.37	83.03 ± 0.43	28.93 ± 0.24
PO7	26.22 ± 0.38	5.73 ± 0.11	74.66 ± 0.75	83.73 ± 0.49	na
Carbon monoxide (ppm)					
PO2	0.98 ± 0.04	1.50 ± 0.03	0.95 ± 0.04	0.70 ± 0.02	0.58 ± 0.02
PO5	na	0.72 ± 0.02	0.60 ± 0.01	na	na
PO6	0.52 ± 0.02	0.75 ± 0.01	0.55 ± 0.01	0.46 ± 0.01	0.45 ± 0.03
PO7	0.47 ± 0.01	0.98 ± 0.08	0.95 ± 0.01	0.78 ± 0.04	na
Nitrogen dioxide (ppb)					
PO2	22.64 ± 0.20	na	na	33.00 ± 0.22	40.38 ± 0.42
PO5	na	na	20.76 ± 0.24	na	na
PO6	21.17 ± 0.61	na	na	20.76 ± 0.52	15.2 ± 0.33
PO7	15.31 ± 0.36	29.12 ± 0.52	na	25.06 ± 0.63	na
Nitric oxide (ppb)					
PO2	33.07 ± 0.76	na	na	21.93 ± 0.98	na
PO5	na	na	8.70 ± 0.51	na	na
PO6	13.74 ± 0.91	na	na	8.42 ± 0.19	na
PO7	6.54 ± 0.38	20.09 ± 0.87	na	na	na
Sulfur dioxide (ppb)					
PO2	5.12 ± 0.09	6.78 ± 0.27	4.91 ± 0.30	1.63 ± 0.06	1.24 ± 0.07
PO5	na	3.15 ± 0.06	2.02 ± 0.14	na	na
PO6	1.75 ± 0.04	0.32 ± 0.12	0.94 ± 0.03	0.47 ± 0.03	1.08 ± 0.04
PO7	0.94 ± 0.03	4.33 ± 0.17	5.3 ± 0.20	2.51 ± 0.06	na

Note: no SE for PM since only one data point available

na = data not available

Table B-2: Plasma Fibrinogen Concentrations For Asthmatic Subjects Individual Data

Subject/Sample	Fibrinogen (mg/dl)				
	FA	CAP	O_3	CAP+O_3	Max CAP
PO2					
pre-exposure	174	183	174	177	240
post-exposure	183	200	190	180	234
post-exercise	183	200	183	167	260
24-hr post-exposure	169	228	177	167	285
PO5					
pre-exposure	na	213	193	na	na
post-exposure	na	234	197	na	na
post-exercise	na	na[1]	186	na	na
24-hr post-exposure	na	na[1]	200	na	na
PO6					
pre-exposure	218	200	169	162	177
post-exposure	197	213	169	183	162
post-exercise	209	213	158	169	169
24-hr post-exposure	223	197	158	160	180
PO7					
pre-exposure	193	172	193	172	na
post-exposure	180	177	102	99	na
post-exercise	167	160	150	148	na
24-hr post-exposure	180	167	172	169	na

Where: FA = filtered air; CAP = 60 $\mu g/m^3$ concentrated ambient $PM_{2.5}$; O_3 = 80 ppb ozone,
Max CAP = 120 $\mu g/m^3$ concentrated ambient $PM_{2.5}$

na = data not available, exposure not conducted

na[1] = data not available due to the presence of clots in the plasma sample

Table B-3
HRV Parameters for Asthmatic Subjects During Exposure (120 minutes)

Target Exposure	LF power (ms^2)	HF power (ms^2)	LF/HF ratio (unitless)	HR (1st hour) (beats/min)	HR (2nd hour) (beats/min)	SDNN (ms)	rMSDD (ms)	pNN50 (%)
PO2								
FA	1476	1478	1.0	75	76	99	60	32.5
CAP	1479	1729	0.9	70	87	98	61	33.7
O_3	2605	2701	1.0	70	71	145	102	63.7
CAP+O_3	1228	1602	0.8	80	92	106	67	37.2
Max CAP	1175	1280	0.9	76	76	89	69	32.3
PO5								
FA	na	na	na	na	na	na	na	na
CAP	na	na	na	na	na	na	na	na
O_3	1310	634	2.1	73	87	105	45	24.1
CAP+O_3	na	na	na	na	na	na	na	na
Max CAP	na	na	na	na	na	na	na	na
PO6								
FA	746	172	4.3	88	94	63	21	3.0
CAP	615	199	3.1	92	98	46	22	3.4
O_3	624	134	4.7	102	113	56	17	1.6
CAP+O_3	1129	373	3.0	92	101	67	28	7.1
Max CAP	na	na	na	na	na	na	na	na
PO7								
FA	1270	598	2.1	82	88	90	39	17.5
CAP	1163	1139	1.0	80	61	108	60	38.9
O_3	1334	1302	1.0	57	77	113	66	45.0
CAP+O_3	1028	771	1.3	65	64	95	52	33.5
Max CAP	na	na	na	na	na	na	na	na

Where: FA = filtered air; CAP = 60 $\mu g/m^3$ concentrated ambient $PM_{2.5}$; O_3 = 80 ppb ozone,

Max CAP = 120 $\mu g/m^3$ concentrated ambient $PM_{2.5}$

Where: LF = low frequency; HF = high frequency; SDNN = standard deviation of NN intervals; rMSDD = square root of mean squared differences of successive NN intervals; pNN50 = proportion of cycles where the difference between adjacent cycles is > 50 ms

na = data not available

Table B-4
HRV Parameters for Asthmatic Subjects During Exercise (30-minutes)

Target Exposure	LF power (ms^2)	HF power (ms^2)	LF/HF ratio (unitless)	Heart Rate (beats/min)	SDNN (ms)	rMSDD (ms)	pNN50 (%)
PO2							
FA	16	7	2.3	na	101	17	2.2
CAP	18	5	3.8	na	65	10	0.2
O_3	22	37	0.6	na	110	29	7.1
CAP+O_3	23	11	2.1	na	98	18	2.0
Max CAP	12	7	1.8	na	76	13	0.4
PO5							
FA	na	na	na	na	na	na	na
CAP	na	na	na	na	na	na	na
O_3	69	8	8.4	na	97	15	1.7
CAP+O_3	na	na	na	na	na	na	na
Max CAP	na	na	na	na	na	na	na
PO6							
FA	56	38	1.5	na	49	13	0.8
CAP	36	19	1.8	na	63	20	2.9
O_3	33	16	2.0	na	44	13	0.6
CAP+O_3	43	21	2.1	na	49	12	0.6
Max CAP	na	na	na	na	na	na	na
PO7							
FA	46	35	1.3	na	73	16	2.1
CAP	28	29	1.0	na	96	17	2.3
O_3	45	50	0.9	na	98	20	3.5
CAP+O_3	45	37	1.2	na	88	18	2.1
Max CAP	na	na	na	na	na	na	na

Where: FA = filtered air; CAP = 60 $\mu g/m^3$ concentrated ambient $PM_{2.5}$; O_3 = 80 ppb ozone,
Max CAP = 120 $\mu g/m^3$ concentrated ambient $PM_{2.5}$

Where: LF = low frequency; HF = high frequency; SDNN = standard deviation of NN intervals; rMSDD = square root of mean squared differences of successive NN intervals; pNN50 = proportion of cycles where the difference between adjacent cycles is > 50 ms

na = data not available

Table B-5: Nasal Lavage Total Cell Counts and Percent Differentials For Asthmatic Subjects - Individual Data

Target Exposure	Sampling Time	Cell Viability (%)	Total cell count (10^3 cells/ml)	Epithelial Cells (%)	Neutrophils (%)	Macrophages (%)	Eosinophils (%)	Lymphocytes (%)
PO2								
FA	pre-exposure	71	151	na	na	na	na	na
	post-exposure	78	831	5	85	0.3	8.5	1.3
	24 hr post	76	249	39	48	0.3	11.3	1.8
CAP	pre-exposure	89	811	2	91	0.5	6.0	0.5
	post-exposure	73	351	14	47	1.2	30.6	7.1
	24 hr post	93	540	1	85	0.3	13.0	1.0
O_3	pre-exposure	92	280	20	76	0.3	2.3	2.3
	post-exposure	79	298	56	40	0.5	3.8	0.5
	24 hr post	72	1415	19	76	0.0	5.0	1.0
CAP+O_3	pre-exposure	91	873	10	90	0.0	0.8	0.0
	post-exposure	77	571	17	81	0.0	0.8	1.0
	24 hr post	61	1320	40	59	0.0	0.3	0.3
Max CAP	pre-exposure	93	695	5	92	0.5	1.8	0.5
	post-exposure	97	433	7	87	0.3	6.8	0.0
	24 hr post	91	493	7	84	0.3	8.3	1.0
PO5								
CAP	pre-exposure	64	56	na	na	na	na	na
	post-exposure	33	20	na	na	na	na	na
	24 hr post	58	691	54	27	1.0	18.3	0.3
O_3	pre-exposure	39	800	89	7	1.0	2.3	1.0
	post-exposure	89	78	40	51	0.0	8.3	1.0
	24 hr post	98	113	8	52	0.0	38.0	2.6
PO6								
FA	pre-exposure	57	476	63	37	0.3	0.5	0.0
	post-exposure	60	695	46	53	0.0	0.3	0.3
	24 hr post	88	204	17	80	0.3	1.3	1.5
CAP	pre-exposure	55	1184	60	39	0.0	0.0	1.0
	post-exposure	87	1460	7	92	0.8	0.0	0.3
	24 hr post	83	791	10	88	0.8	1.5	0.8
O_3	pre-exposure	79	476	28	70	0.3	1.8	1.0
	post-exposure	75	564	37	60	0.3	2.3	0.3
	24 hr post	68	720	36	58	0.3	1.0	4.3
CAP+O_3	pre-exposure	86	1171	8	92	0.0	0.0	1.0
	post-exposure	83	822	11	89	0.0	0.3	0.3
	24 hr post	79	804	15	83	1.0	0.5	0.3
Max CAP	pre-exposure	31	284	81	15	0.5	2.5	1.3
	post-exposure	70	249	36	55	0.8	7.3	7.8
	24 hr post	69	120	41	40	1.5	17.3	1.0
PO7								
FA	pre-exposure	71	38	na	na	na	na	na
	post-exposure	57	67	44	48	1.0	5.8	75.0
	24 hr post	83	147	na	46	0.8	14.3	0.0
CAP	pre-exposure	96	122	7	90	0.3	2.3	0.5
	post-exposure	78	60	5	90	0.0	3.5	1.8
	24 hr post	92	53	na	82	1.0	13.3	2.0
O_3	pre-exposure	96	49	9	90	0.0	2.0	0.0
	post-exposure	62	831	33	60	0.8	5.5	1.5
	24 hr post	96	280	na	50	1.0	38.3	4.0
CAP+O_3	pre-exposure	67	20	na	na	na	na	na
	post-exposure	85	58	2	92	0.0	5.3	0.8
	24 hr post	92	211	na	37	2.8	29.0	4.8

na= sample inadequate for processing

Table B-6: Protein Concentration in Nasal Lavage Fluid and Raw Sputum of Asthmatic Subjects

Target Exposure	Sampling Time	Nasal Lavage Protein Concentration (μg/ml)	Raw Sputum Protein Concentration (μg/ml)
PS2			
FA	pre-exposure	922	2800
	post-exposure	1669	1191
	24-hr post-exposure	1276	1080
CAP	pre-exposure	1486	988
	post-exposure	986	1213
	24-hr post-exposure	1026	na
O_3	pre-exposure	701	3867
	post-exposure	1412	1158
	24-hr post-exposure	1224	2725
CAP+O_3	pre-exposure	1535	2800
	post-exposure	980	2287
	24-hr post-exposure	1014	2424
Max CAP	pre-exposure	1355	2842
	post-exposure	1282	2561
	24-hr post-exposure	962	3260
PS5			
CAP	pre-exposure	1204	na
	post-exposure	839	na
	24-hr post-exposure	942	2274
O_3	pre-exposure	1060	2741
	post-exposure	714	na
	24-hr post-exposure	1054	1289
PS6			
FA	pre-exposure	1684	1021
	post-exposure	2073	1093
	24-hr post-exposure	750	1370
CAP	pre-exposure	1799	854
	post-exposure	1715	1054
	24-hr post-exposure	1072	1334
O_3	pre-exposure	1418	94
	post-exposure	1477	-98
	24-hr post-exposure	1751	137
CAP+O_3	pre-exposure	2858	450
	post-exposure	1502	440
	24-hr post-exposure	1178	665
Max CAP	pre-exposure	1264	1377
	post-exposure	1937	1556
	24-hr post-exposure	1316	1530
PS7			
FA	pre-exposure	176	2633
	post-exposure	292	1755
	24-hr post-exposure	337	1876
CAP	pre-exposure	304	3449
	post-exposure	514	2379
	24-hr post-exposure	636	2065
O_3	pre-exposure	243	2392
	post-exposure	444	1726
	24-hr post-exposure	450	2078
CAP+O_3	pre-exposure	196	3129
	post-exposure	220	2193
	24-hr post-exposure	564	1866

Note: For sputum samples, three aliquots of each sample were analyzed, average concentration shown

Note: For nasal lavage samples, two aliquots of each sample were analyzed, average concentration shown

Table B-7: Sputum Total Cell Counts and Percent Differentials for Asthmatic Subjects

Target Exposure	Sampling Time	Cell Viability (%)	Total cell count (10^3 cells/ml)	Epithelial Cells (%)	Neutrophils (%)	Macrophages (%)	Eosinophils (%)	Lymphocytes (%)
PO2								
FA	pre-exposure	32	498	0.75	14.00	55.50	14.75	15.00
	post-exposure	19	1700	na	na	na	na	na
	24 hr post	70	247	1.50	40.00	42.50	13.25	2.75
CAP	pre-exposure	58	227	1.00	23.50	46.25	24.75	4.50
	post-exposure	41	791	0.25	32.00	50.75	13.25	3.75
	24 hr post	55	5331	0.00	17.25	73.75	3.50	5.50
O_3	pre-exposure	47	169	8.50	32.50	46.00	7.50	5.50
	post-exposure	26	260	1.00	8.25	88.00	2.75	0.00
	24 hr post	44	191	1.25	23.00	72.25	1.25	2.25
CAP+O_3	pre-exposure	48	167	2.25	21.75	68.50	3.50	4.00
	post-exposure	36	376	1.00	6.25	88.75	2.50	1.50
	24 hr post	56	116	2.25	17.00	76.75	2.50	1.50
High CAP	pre-exposure	47	113	0.25	18.75	57.75	17.50	5.75
	post-exposure	37	467	na	na	na	na	na
	24 hr post	62	227	1.25	25.75	51.00	20.75	1.25
PO5								
CAP	pre-exposure	44	229	3.75	8.75	80.75	5.25	1.50
	post-exposure	67	109	1.50	31.00	44.25	20.50	2.75
	24 hr post	47	735	0.75	17.50	41.00	38.00	2.75
O_3	pre-exposure	76	253	0.25	7.50	41.75	47.50	3.00
	post-exposure	na	na	na	na	na	na	na
	24 hr post	35	329	na	na	na	na	na
PO6								
FA	pre-exposure	61	220	0.50	28.75	59.75	5.75	5.25
	post-exposure	55	229	0.25	56.50	37.00	3.00	3.25
	24 hr post	67	209	0.50	83.75	14.25	0.50	1.00
CAP	pre-exposure	na	na	na	na	na	na	na
	post-exposure	61	744	0.25	57.50	30.25	8.75	3.25
	24 hr post	64	178	0.25	36.75	57.50	4.25	1.25
O_3	pre-exposure	52	111	10.00	6.75	74.25	7.50	1.50
	post-exposure	56	147	9.75	11.75	62.00	13.50	3.00
	24 hr post	91	98	2.25	41.75	45.75	5.75	4.50
CAP+O_3	pre-exposure	59	213	0.75	17.50	72.75	3.25	5.75
	post-exposure	71	424	0.00	38.75	51.00	3.25	7.00
	24 hr post	80	133	0.50	46.75	40.00	3.25	9.50
High CAP	pre-exposure	42	118	na	na	na	na	na
	post-exposure	39	113	na	na	na	na	na
	24 hr post	55	93	0.75	24.50	62.50	5.50	6.75
PO7								
FA	pre-exposure	75	302	0.75	25.25	67.00	5.25	1.75
	post-exposure	80	478	0.00	80.25	13.25	4.25	2.25
	24 hr post	85	144	0.25	45.75	35.25	12.75	6.00
CAP	pre-exposure	82	316	0.75	33.25	58.25	5.25	2.50
	post-exposure	88	280	0.50	54.50	37.75	3.00	4.00
	24 hr post	85	729	0.25	52.00	39.75	4.25	3.75
O_3	pre-exposure	75	591	0.00	64.00	31.75	2.50	1.75
	post-exposure	50	595	0.75	29.50	63.25	2.25	4.25
	24 hr post	69	364	0.25	33.25	59.00	4.50	3.00
CAP+O_3	pre-exposure	73	222	0.25	11.25	73.00	12.75	2.50
	post-exposure	63	420	0.00	38.25	45.50	14.25	2.00
	24 hr post	61	347	0.50	25.75	61.00	9.50	3.25

na= sample inadequate for processing

Table C-1: Variability in Environmental Parameters During Exposure for Healthy Subjects

Subject/Sample	FA (mean ± SD)	low CAP (mean ± SD)	mid CAP (mean ± SD)	high CAP (mean ± SD)
$PM_{2.5}$ gravimetric ($\mu g/m^3$)	na	31.5 ± 7.9	52.9 ± 33.9	92.1 ± 24.6
Ozone (ppb)	9.3 ± 11.6	10.9 ± 9.5	10.5 ± 10.3	7.0 ± 2.6
Carbon monoxide (ppm)	na	0.2 ± 0.1	0.3 ± 0.2	0.5 ± 0.4
Nitrogen dioxide (ppb)	na	17.4 ± 9.2	18.8 ± 7.9	25.2 ± 5.5
Nitric oxide (ppb)	9.1 ± 7.7	13.4 ± 9.1	16.8 ± 14.2	32.1 ± 30.6
Sulfur dioxide (ppb)	5.6 ± 4.9	0.6 ± 0.4	2.4 ± 3.2	2.0 ± 1.3

na = data not available

Table C-2: Variability in Plasma Fibrinogen and Factor VII Concentrations in Healthy Subjects (mean ± SD)

Subject/Sample	Fibrinogen (mg/dl)				Factor VII (%)			
	FA	Low CAP	Mid CAP	High CAP	FA	Low CAP	Mid CAP	High CAP
pre-exposure	271 ± 56	280 ± 60	253 ± 45	286 ± 85	94 ± 4	96 ± 2	94 ± 3	100 ± 5
post-exposure	286 ± 65	272 ± 81	258 ± 47	296 ± 100	98 ± 5	99 ± 3	99 ± 3	104 ± 5
post-exercise	279 ± 68	279 ± 71	254 ± 43	319 ± 120	100 ± 9	98 ± 1	106 ± 11	98 ± 2
24-hr post-exposure	279 ± 54	288 ± 106	265 ± 45	271 ± 105	98 ± 5	99 ± 9	100 ± 6	102 ± 3

na = no data available

Table C-3: Variability in HRV Parameters in Healthy Subjects for Exposure Period (mean + SD)

Target Exposure	LF power $(beats/min)^2$	HF power $(beats/min)^2$	LF/HF ratio (unitless)	Ave Heart Rate (beats/min)	SDNN (beats/min)
FA	8.38 ± 6.14	4.07 ± 2.14	$1.97 + 0.52$	$65 + 12$	$4.65 + 1.34$
Low CAP	7.70 ± 4.60	5.93 ± 2.10	1.29 ± 0.51	64 ± 9	5.09 ± 0.96
Mid Cap	9.62 ± 6.92	5.10 ± 2.79	1.87 ± 0.34	66 ± 13	5.11 ± 1.72
High CAP	8.80 ± 4.89	4.82 ± 2.47	2.05 ± 0.81	69 ± 15	5.04 ± 1.37

Table C-4: Variability in HRV Parameters in Healthy Subjects for Exercise Period (mean + SD)

Target Exposure	LF power $(beats/min)^2$	HF power $(beats/min)^2$	LF/HF ratio (unitless)	Ave Heart Rate (beats/min)	SDNN (beats/min)
FA	3.66 ± 2.64	1.27 ± 1.45	3.96 ± 1.59	124 ± 2	3.19 ± 0.77
Low CAP	4.36 ± 2.60	2.63 ± 1.89	2.56 ± 1.62	125 ± 5	4.86 ± 1.50
Mid Cap	4.36 ± 2.14	1.62 ± 1.36	3.69 ± 2.70	125 ± 3	3.91 ± 0.81
High CAP	3.26 ± 1.51	2.45 ± 3.13	3.03 ± 1.95	124 ± 2	4.10 ± 1.56

Table C-5: Variability in Nasal Lavage Parameters in Healthy Subjects (mean ± SD)

Target CAP	Sampling Time	Cell Viability (%)	Total cell count (10^3 cells/ml)	Epithelial Cells (%)	Neutrophils (%)	Macrophages (%)	Eosinophils (%)	Lymphocytes (%)
FA	pre-exposure	67 ± 18	322 ± 252	66 ± 26	31 ± 24	0.4 ± 0.4	1.5 ± 2.2	0.6 ± 1.0
	post-exposure	66 ± 15	380 ± 194	63 ± 22	32 ± 21	1.0 ± 0.4	2.1 ± 3.8	2.0 ± 2.0
	24 hr post	58 ± 16	441 ± 452	71 ± 15	27 ± 14	0.7 ± 0.1	0.9 ± 1.0	0.6 ± 0.6
Low CAP	pre-exposure	62 ± 11	462 ± 405	83 ± 12	14 ± 11	0.3 ± 0.3	0.4 ± 0.4	1.8 ± 1.1
	post-exposure	71 ± 17	1199 ± 1606	51 ± 25	45 ± 23	1.1 ± 1.2	1.0 ± 1.0	2.3 ± 0.9
	24 hr post	57 ± 12	438 ± 491	41 ± 21	53 ± 16	1.1 ± 0.9	3.9 ± 6.6	0.8 ± 1.0
Mid CAP	pre-exposure	53 ± 14	964 ± 651	70 ± 18	26 ± 17	0.8 ± 0.8	2.4 ± 4.4	1.5 ± 1.1
	post-exposure	66 ± 19	511 ± 492	51 ± 26	46 ± 24	0.7 ± 0.7	1.2 ± 1.9	0.8 ± 1.0
	24 hr post	64 ± 16	808 ± 556	60 ± 13	36 ± 14	1.4 ± 0.6	1.1 ± 1.9	1.1 ± 0.7
High CAP	pre-exposure	54 ± 24	1033 ± 959	47 ± 20	50 ± 21	0.3 ± 0.0	1.2 ± 0.1	1.4 ± 1.2
	post-exposure	52 ± 14	704 ± 220	62 ± 23	37 ± 23	0.4 ± 0.5	0.8 ± 0.9	0.6 ± 0.1
	24 hr post	51 ± 16	719 ± 396	34 ± 11	62 ± 9	1.2 ± 0.3	1.9 ± 1.7	1.2 ± 1.2

Table C-6: Variability in Protein Concentrations in Healthy Subjects (mean ± SD)

Target CAP Exposure	Sampling Time	Nasal Lavage Protein Concentration (μg/ml)	Raw Sputum Protein Concentration (μg/ml)
FA	pre-exposure	543 ± 190	na
	post-exposure	594 ± 217	1872 ± 694
	24-hr post-exposure	673 ± 325	2236 ± 1031
Low CAP	pre-exposure	584 ± 134	na
	post-exposure	638 ± 160	2227 ± 1235
	24-hr post-exposure	831 ± 71	2986 ± 1720
Mid CAP	pre-exposure	654 ± 81	na
	post-exposure	652 ± 213	2865 ± 1959
	24-hr post-exposure	732 ± 138	2804 ± 1259
High CAP	pre-exposure	567 ± 310	na
	post-exposure	611 ± 267	2086 ± 265
	24-hr post-exposure	675 ± 308	2561 ± 230

na = no data available

Table C-7: Variability in Sputum Parameters in Healthy Subjects (mean ± SD)

Target CAP Exposure	Cell Viability (%) post	Cell Viability (%) 24-hour	Total cell count (10^3 cells/ml) post	Total cell count (10^3 cells/ml) 24-hour	Neutrophils (%) post	Neutrophils (%) 24-hour	Macrophages (%) post	Macrophages (%) 24-hour	Eosinophils (%) post	Eosinophils (%) 24-hour	Lymphocytes (%) post	Lymphocytes (%) 24-hour
FA	71 ± 5	75 ± 5	657 ± 366	559 ± 313	45 ± 38	46 ± 26	51 ± 39	48 ± 25	0.9 ± 1.4	0.9 ± 0.7	2.5 ± 1.0	4.4 ± 1.5
Low CAP	64 ± 9	69 ± 8	673 ± 339	3320 ± 4241	28 ± 24	53 ± 28	68 ± 24	43 ± 27	0.2 ± 0.2	0.3 ± 0	3.4 ± 1.5	3.6 ± 1.4
Mid CAP	59 ± 6	69 ± 7	659 ± 436	960 ± 316	37 ± 12	52 ± 16	59 ± 13	43 ± 16	0.4 ± 0.5	0.4 ± 0.5	3.8 ± 0	4.8 ± 0.5
High CAP	65 ± 11	na	765 ± 276	na	32 ± 13	na	64 ± 10	na	0.3 ± 0.4	na	4.1 ± 2.5	na

Sampling Times are: post = post-exposure and 24-hour = 24 hours post-exposure
na = data not available

Table D-1: Variability in Environmental Parameters During Exposure for Asthmatic Subjects

Subject/Sample	FA (mean ± SD)	CAP (mean ± SD)	O_3 (mean ± SD)	CAP + O_3 (mean ± SD)	Max CAP (mean ± SD)
$PM_{2.5}$ gravimetric ($\mu g/m^3$)	1.2 ± 1.2	46.7 ± 10.3	3.9 ± 2.6	80.9 ± 38.2	na
Ozone (ppb)	16.2 ± 12.4	2.9 ± 2.2	80.4 ± 6.1	83.2 ± 0.4	na
Carbon monoxide (ppm)	0.7 ± 0.3	1.0 ± 0.4	0.8 ± 0.2	0.6 ± 0.2	na
Nitrogen dioxide (ppb)	19.7 ± 3.9	na	na	26.3 ± 6.2	na
Nitric oxide (ppb)	17.8 ± 13.7	na	na	na	na
Sulfur dioxide (ppb)	2.6 ± 2.2	3.6 ± 2.7	3.3 ± 2.1	1.5 ± 1.0	na

na = data not available

Table D-2: Variability in Plasma Fibrinogen Concentrations in Asthmatic Subjects (mean ± SD)

Subject/Sample	Fibrinogen (mg/dl)				
	FA	CAP	O_3	CAP+O_3	Max CAP
pre-exposure	195 ± 22	192 ± 18	182 ± 13	170 ± 8	na
post-exposure	187 ± 9	206 ± 24	165 ± 43	154 ± 48	na
post-exercise	186 ± 21	191 ± 28	169 ± 18	161 ± 12	na
24-hr post-exposure	191 ± 29	197 ± 31	177 ± 17	165 ± 5	na

na = data not available

Table D-3: Variability in HRV Parameters in Asthmatic Subjects for Exposure Period (mean + SD)

Target Exposure	LF power (ms^2)	HF power (ms^2)	LF/HF ratio (unitless)	HR (1st hour) (beats/min)	HR (2nd hour) (beats/min)	SDNN (ms)	rMSDD (ms)	pNN50 (%)
FA	1164 ± 377	749 ± 666	2 ± 2	82 ± 7	86 ± 9	84 ± 19	40 ± 20	18 ± 15
CAP	1086 ± 437	1022 ± 772	1.6 ± 1.2	81 ± 11	82 ± 19	84 ± 33	48 ± 22	25 ± 19
O_3	1468 ± 826	1192 ± 1114	2.2 ± 1.7	76 ± 19	87 ± 19	105 ± 37	58 ± 36	34 ± 27
CAP+O_3	1128 ± 100	915 ± 627	1.7 ± 1.2	79 ± 14	86 ± 19	89 ± 20	49 ± 20	26 ± 16
Max CAP	na	na	na	na	na	na	na	na

na = data not available

Table D-4: Variability in HRV Parameters in Asthmatic Subjects for Exercise Period (mean + SD)

Target Exposure	LF power (ms^2)	HF power (ms^2)	LF/HF ratio (unitless)	Heart Rate (beats/min)	SDNN (ms)	rMSDD (ms)	pNN50 (%)
FA	40 ± 21	27 ± 17	1.7 ± 0.5	na	74 ± 26	15 ± 2	1.7 ± 0.8
CAP	28 ± 9	18 ± 12	2.2 ± 1.4	na	75 ± 19	16 ± 5	1.8 ± 1.4
O_3	42 ± 20	28 ± 19	3.0 ± 3.6	na	87 ± 29	19 ± 7	3.2 ± 2.8
CAP+O_3	37 ± 12	23 ± 13	1.8 ± 0.5	na	78 ± 26	16 ± 3	1.6 ± 0.8
Max CAP	na	na	na	na	na	na	na

na = data not available

Table D-5: Variability in Nasal Lavage Parameters For Asthmatic Subjects (mean ± SD)

Target Exposure	Sampling Time	Cell Viability (%)	Total cell count (10^3 cells/ml)	Epithelial Cells (%)	Neutrophils (%)	Macrophages (%)	Eosinophils (%)	Lymphocytes (%)
FA	pre-exposure	66 ± 8	221 ± 227	na	na	na	na	na
	post-exposure	65 ± 11	531 ± 408	32 ± 23	62 ± 20	0.4 ± 0.5	4.8 ± 4.2	25.5 ± 42.9
	24 hr post	82 ± 6	200 ± 51	na	58 ± 19	0.4 ± 0.3	8.9 ± 6.8	1.1 ± 0.9
CAP	pre-exposure	69 ± 18	684 ± 575	31 ± 41	65 ± 36	0.3 ± 0.4	3.0 ± 4.2	0.8 ± 0.4
	post-exposure	68 ± 24	473 ± 674	9 ± 5	76 ± 25	0.6 ± 0.6	11.4 ± 16.7	3.0 ± 3.6
	24 hr post	82 ± 16	519 ± 327	21 ± 28	70 ± 29	0.8 ± 0.4	11.5 ± 7.1	1.0 ± 0.7
O_3	pre-exposure	70 ± 28	518 ± 263	45 ± 38	51 ± 38	0.5 ± 0.4	2.1 ± 0.3	1.4 ± 0.7
	post-exposure	76 ± 11	443 ± 326	41 ± 10	53 ± 9	0.4 ± 0.3	5.0 ± 2.6	0.8 ± 0.6
	24 hr post	84 ± 16	632 ± 581	21 ± 14	59 ± 12	0.3 ± 0.5	20.6 ± 20.4	3.0 ± 1.5
CAP+O_3	pre-exposure	81 ± 13	688 ± 597	na	na	na	na	na
	post-exposure	82 ± 4	484 ± 390	10 ± 8	87 ± 5	0 ± 0	2.1 ± 2.8	0.7 ± 0.4
	24 hr post	77 ± 16	778 ± 555	na	60 ± 23	1.3 ± 1.4	9.9 ± 16.5	1.8 ± 2.6
Max CAP	pre-exposure	na	na	na	na	na	na	na
	post-exposure	na	na	na	na	na	na	na
	24 hr post	na	na	na	na	na	na	na

na = data not available

Table D-6: Variability in Protein Concentrations in Asthmatic Subjects (mean ± SD)

Target Exposure	Sampling Time	Nasal Lavage Protein Concentration (μg/ml)	Raw Sputum Protein Concentration (μg/ml)
FA	pre-exposure	928 ± 754	2151 ± 982
	post-exposure	1345 ± 934	1346 ± 358
	24-hr post-exposure	788 ± 470	1442 ± 403
CAP	pre-exposure	1198 ± 644	1764 ± 1461
	post-exposure	1014 ± 508	1549 ± 723
	24-hr post-exposure	919 ± 196	1891 ± 494
O_3	pre-exposure	855 ± 502	2273 ± 1583
	post-exposure	1012 ± 512	na
	24-hr post-exposure	1120 ± 536	1557 ± 1114
CAP+O_3	pre-exposure	1529 ± 1331	2126 ± 1461
	post-exposure	901 ± 645	1640 ± 1040
	24-hr post-exposure	919 ± 318	1652 ± 899
Max CAP	pre-exposure	na	na
	post-exposure	na	na
	24-hr post-exposure	na	na

na = data not available

Table D-7: Variability in Sputum Parameters in Asthmatic Subjects (mean ± SD)

Target Exposure	Sampling Time	Cell Viability (%)	Total cell count (10^3 cells/ml)	Epithelial Cells (%)	Neutrophils (%)	Macrophages (%)	Eosinophils (%)	Lymphocytes (%)
FA	pre-exposure	56 ± 22	340 ± 143	0.6 ± 0.1	23 ± 8	61 ± 6	9 ± 5	7 ± 7
	post-exposure	51 ± 31	802 ± 787	na	na	na	na	na
	24 hr post	74 ± 10	200 ± 52	0.8 ± 0.7	57 ± 24	31 ± 15	9 ± 7	3.3 ± 2.5
CAP	pre-exposure	61 ± 19	257 ± 51	1.8 ± 1.7	22 ± 12	62 ± 18	12 ± 11	2.8 ± 1.5
	post-exposure	64 ± 19	481 ± 339	0.6 ± 0.6	44 ± 14	41 ± 9	11 ± 7	3.4 ± 0.6
	24 hr post	63 ± 16	1743 ± 2406	0.3 ± 0.3	31 ± 17	53 ± 16	13 ± 17	3.3 ± 1.8
O_3	pre-exposure	63 ± 15	281 ± 215	5 ± 5	28 ± 27	48 ± 18	16 ± 21	2.9 ± 1.8
	post-exposure	44 ± 16	334 ± 233	4 ± 5	17 ± 11	71 ± 15	6 ± 6	2.4 ± 2.2
	24 hr post	60 ± 25	246 ± 124	1 ± 1	33 ± 9	59 ± 13	4 ± 2	3 ± 1
CAP+O_3	pre-exposure	60 ± 13	201 ± 30	1 ± 1	17 ± 5	71 ± 3	7 ± 5	4 ± 2
	post-exposure	57 ± 18	408 ± 27	0.3 ± 0.6	28 ± 19	62 ± 24	7 ± 7	3.5 ± 3.0
	24 hr post	66 ± 13	198 ± 129	1 ± 1	30 ± 15	59 ± 18	5 ± 4	5 ± 4
Max CAP	pre-exposure	na	na	na	na	na	na	na
	post-exposure	na	na	na	na	na	na	na
	24 hr post	na	na	na	na	na	na	na

na = data not available