EFFECTS OF AIR POLLUTANTS ON ENDOTHELIAL FUNCTION

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ABSTRACT OF THE DISSERTATION

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Background: Substantial evidence links exposure to particulate matter air pollution with cardiovascular morbidity and mortality. Endothelial dysfunction has been recently investigated as a potential mechanism by which an exposure to air pollutants for a short period of time can cause adverse cardiovascular events.

Methods: We examined the intra-day reproducibility of EndoPAT measurements obtained 2.5 hours apart and the inter-day reproducibility of EndoPAT measurements obtained 1 week apart in a group of young and healthy volunteers. We also examined the acute changes in EndoPAT measurements and plasma nitrite concentration following a 2-hour exposure to diesel exhaust (DE) and secondary organic aerosol (SOA) compared to clean air (CA) in a controlled environmental facility as well as changes in these markers associated with increases in 5 ambient air pollutant concentrations, namely particulate matter less than 2.5 micrometers in diameter ($PM_{2.5}$), carbon monoxide (CO), sulphur dioxide (SO_2), nitrogen dioxide (NO_2) and ozone (O_3) on the preceding 7 days.

Results: In Aim 1, the intra-class correlation coefficients (ICC) for the PAT ratio obtained 2.5 hours apart were -0.07 and 0.40 in the pilot and CA exposure study,

respectively, and the ICC for the PAT ratio obtained a week apart was 0.27, indicating a low intra-day and inter-day reproducibility. In Aim 2, the plasma nitrite concentration showed a greater decrease following the exposure to DE and SOA compared to CA. The mean PAT ratios showed an increase from pre to post-exposure to DE, SOA, and CA. In Aim 3, each interquartile range increase in the mean $PM_{2.5}$ and CO concentration in the first 24 hours before the endothelial function measurement was associated with an increase in the plasma nitrite concentration (17.1 and 17.2% respectively).

Conclusions: The EndoPAT device does not produce reliable PAT ratios 2.5 hours apart and one week apart in a young population. The increase in the plasma nitrite associated with the increase in the ambient air pollutants in the first 24 hours suggests a potential role of systemic inflammation via stimulation of inducible nitric oxide synthase enzyme and generation of nitric oxide within a short period of time.

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LIST OF ABBREVIATIONS

PM Particulate matter

BAFMD Flow mediated dilation of brachial artery

CVD Cardiovascular disease

PAT Peripheral arterial tonometry

DE Diesel exhaust

CA Clean air

SOA Secondary organic aerosols

BAD Brachial artery diameter

ICC Intra class correlation

CEF Controlled environmental facility

PWA Pulse wave amplitude

CI Confidence interval

SD Standard deviation

CV Coefficient of variation

CAD Coronary artery disease

CHF Congestive heart failure

NO Nitric oxide

eNOS Endothelial nitric oxide synthase

iNOS Inducible nitric oxide synthase

MI Myocardial infarction

EPA Environmental Protection Agency

EOHSI Environmental and Occupational Health Sciences Institute

INTRODUCTION

Air Pollution and Cardiovascular Diseases

Over the past two decades, several epidemiological studies have reported a strong association between exposure to particulate matter (PM) air pollution and cardiovascular morbidity and mortality.[1-11] In addition to PM, exposure to other criteria air pollutants such as carbon monoxide, sulphur dioxide, nitrogen dioxide, elemental carbon, and ozone has been shown to cause increased hospital admissions and deaths due to myocardial infarction (MI), arrhythmia, congestive heart failure (CHF), and angina throughout the industrialized world.[6, 7, 12-19]

Ambient Air pollutants

Air pollution is a heterogeneous mixture of gases and particulate matter. The United States Clean Air Act requires the United States Environmental Protection Agency (USEPA) to set national ambient air quality standards (NAAQS) for common air pollutants, also known as criteria air pollutants. These pollutants are found all over the United States.

a. Particulate Matter (PM)

PM is a complex mixture of solid and liquid particles suspended in air. These particles vary greatly in size and chemical composition. There are two types of particles. Primary particles are emitted directly into the atmosphere, such as diesel soot, whereas secondary particles are generated through physicochemical transformation of gases, such as nitrate and sulfate formation from gaseous nitric acid and sulfur dioxide (SO2), respectively.

The numerous sources of PM include motor vehicle emissions, resuspension of road dust, power generation and other industrial combustion, smelting and other metal processing, agriculture, construction and demolition activities, and residential wood burning.[20, 21] The USEPA regulates ambient air quality by setting standards for PM and is most concerned about particles with a median diameter of less than 10 μm as these particles are readily inhaled and absorbed into the pulmonary vasculature. PM are classified based on their size as follows. PM with a median aerodynamic diameter of < 10 μm are referred to as thoracic particles or PM_{10}. Particles ranging in size from 2.5 to 10 μm are referred to as coarse particles. Fine PM consists of particles less than 2.5 μm ($PM_{2.5}$) and ultrafine PM consists of particles less than 0.1 μm ($PM_{0.1}$).[20] Larger particles such as PM_{10} tend to get deposited in upper airways whereas smaller particles such as $PM_{0.1}$ tend to reach the alveoli.

b. Gaseous Pollutants: The sources and health effects of gaseous criteria air pollutants such as sulphur dioxide, nitrogen dioxide, ozone, and carbon monoxide have been summarized in Table 1.

Biological Mechanisms Explaining the Link between Cardiovascular Diseases and Air Pollutants

Several underlying biologic mechanisms have been proposed and investigated to explain the link between exposure to pollutants and cardiovascular diseases (Figure 1). Soluble constituents of PM and gases can readily cross the pulmonary epithelium and can be absorbed into the systemic circulation via the pulmonary vasculature. These constituents may exert an oxidative effect on heart and blood vessels and promote harmful effects on

cardiovascular systems. These direct effects may be responsible for rapid or hyperacute (within minutes to hours) cardiovascular responses such as MI and arrhythmias following an exposure to PM. [20] Direct activation of pulmonary neural reflexes after inhalation of PM has been suggested to play a role in altering autonomic (sympathetic and parasympathetic) tone.[20, 22] This, in turn, may lead to instability of a vascular plaque or initiation of cardiac arrhythmias. A decrease in heart rate variability [23-26] and changes in blood pressure and resting heart rate [27] have also been postulated as one of the mechanisms by which PM can cause fatal arrhythmias.

Many animal as well as human studies have demonstrated a role of pulmonary and systemic inflammation through the action of cytokines and other soluble mediators generated in the lung following the inhalation of PM.[27-30] Van Eeden et al demonstrated a release of pro-inflammatory cytokines such as tumor necrosis factor-alpha, interleukin-6 (IL-6), IL-1β, and granulocyte-macrophage colony stimulating factor by human alveolar macrophages, when the macrophages were exposed to ambient urban particles.[28] Based on these findings, they further hypothesized that these cytokines enter the systemic circulation and induce a systemic response which may be involved in the pathogenesis of cardiovascular events. An increase in C-reactive protein, an inflammatory marker associated with an exposure to air pollutants as shown by Peters et al[31] and Pope et al[32] further strongly supports a role of systemic inflammation in the pathogenesis of cardiovascular events.

Exposure to PM may lead to alveolar inflammation and this low grade inflammation may then be responsible for activating clotting pathways. It has been shown that several hematological factors such as clotting factors, fibrinogen, and Factor VIII are

produced in response to inflammation resulting in an increase in blood viscosity.[33] Nemmar et al demonstrated an enhanced thrombosis following intravenous or intratracheal administration of synthetic ultrafine particles in a hamster model.[34] This finding suggested roles for the activation of coagulation system and platelets as operating mechanisms. Two other studies explored the role of platelet activation and aggregation in response to exposure to diesel exhaust and ultrafine particles.[35, 36] Ruckerl et al showed that sCD40L, a soluble marker of platelet activation, was significantly increased by roughly 7% within 24-hours of an increase in ambient ultrafine and accumulation mode particles in a panel of male patients with a history of coronary artery disease.[35] In a double-blind randomized cross-over study, Lucking et al showed that a 2-hour exposure to DE increased thrombus formation at 2 and 6 hours following the exposure.[36]

Lastly, endothelial dysfunction can arise as a consequence of systemic inflammation and/or oxidative stress. Cytokines activate endothelial cells and upregulate endothelial cell adhesion molecules. Oxidative stress can result in decreased synthesis of nitric oxide (NO) due to limitation of the redox-sensitive cofactor tetrahydrobiopterin and in decreased bioavailability of NO due to reaction with superoxide. These processes can affect vasoreactivity and due to which, blood vessels may not be able to respond to vasoconstrictor stimuli with compensatory vasodilation. Several studies have explored the possibility of endothelial dysfunction as a potential mechanism linking air pollutants and cardiovascular events.[37-39] For instance, a randomized, double-blind crossover study demonstrated a brachial artery vasoconstriction response following a 2-hour inhalation of mixture of ambient fine particles and ozone as compared to filtered air inhalation among healthy adults.[37] Another controlled exposure study reported a similar finding

following a 2-hour exposure to diesel exhaust (DE) at two different doses. [38] Schneider et al investigated the effects of ambient $PM_{2.5}$ on endothelial function measured using flow-mediated dilation (FMD) in a vulnerable population of diabetic individuals and demonstrated a decrease in FMD in association with $PM_{2.5}$ within the first 24 hours suggesting acute endothelial dysfunction.[39]

At this point, it can be presumed that many of these above mentioned mechanisms may operate concurrently in response to PM inhalation. However, there are inconsistencies in the data and further research is warranted to understand the complexity and inter-dependency of each of these mechanisms. Given this background, the goal of this dissertation was to explore endothelial dysfunction as a potential mechanism by which air pollutants may cause adverse cardiovascular events.

Acute versus Hyperacute Effects of Air Pollutants

The timing of cardiovascular responses to air pollution particles following an exposure may play a key role in understanding relevant biologic mechanisms involved in disease exacerbation. As discussed earlier, air pollution particles may have direct effects on the cardiovascular system and blood and/or indirect effects mediated through pulmonary oxidative stress and systemic inflammatory responses.[20] Direct effects may explain hyperacute responses commonly observed within a few hours of exposure whereas indirect effects may explain acute responses observed either within the same day or a few days following exposure.[20] As stated earlier, several studies have reported an association between the risk of hospitalization for adverse cardiovascular events such as arrhythmias, MI and CHF and exposure to high levels of PM within the same day [2, 7, 8, 16, 40, 41] or a few

days prior to the event.[13, 42] Recently, small but significant increase in pulmonary artery diastolic pressure and right ventricular diastolic pressure associated with an increase in same day mean $PM_{2.5}$ concentration was reported by Rich et al (2008).[43] There is also substantial evidence suggesting that cardiovascular events may be triggered within a few hours of exposure to high concentration of particles.[4, 16, 17, 44] Peters et al investigated this hypothesis in the greater Boston area using a case cross-over approach. They reported that the risk of MI increased by 48% for a 25 $\mu g/m^3$ increase in $PM_{2.5}$ over the preceding 2 hours.[44] Similarly, increased risk of symptom exacerbation of CHF in response to exposure to $PM_{2.5}$ was observed in another case cross-over study within 8 hours prior to the onset of symptoms of CHF.[4] Additional studies by Rich et al (2006) reported increased risks of arrhythmias associated with exposure to ambient air pollution within 1 and 24 hours.[16-17] Findings from all above-mentioned studies emphasize the potential of rapid action of air pollutants on the cardiovascular system and suggest that further studies are warranted to explore the rapid and hyperacute actions of underlying mechanisms.

Endothelial Dysfunction

The endothelium, a barrier between vessel lumen and surrounding tissues, is a thin layer of cells that line the interior surface of blood vessels. These cells form an interface between circulating blood and the rest of the blood vessel wall. The basic function of endothelial cells is to regulate blood pressure via vasodilation (dilation of blood vessels) or vasoconstriction (constriction of blood vessels) and to regulate the process of blood clotting. Endothelial dysfunction is defined as the inability of blood vessels to dilate fully in response to an appropriate stimulus. It is a hallmark for vascular diseases, and is often

considered as a key early event in the development of atherosclerosis. Impaired endothelial function is seen in patients with coronary artery disease and diabetes mellitus as well as hypertension. It can be measured using several invasive and non-invasive procedures. Although techniques such as coronary angiography and strain gauge plethysmography are considered to be the diagnostic gold standard for the evaluation of endothelial function, these techniques are invasive and risky. Several non-invasive methods that are commonly used are as follows: (1) measurement of morphologic and mechanical characteristics of the vascular wall (e.g. intima-media thickness, compliance, distensibility, and remodeling indexes), (2) determination of soluble endothelial function markers (e.g Von Willebrand factor, plasminogen activator), and (3) measurement of the endothelium-dependent regulation of vascular tone at focal sites of circulation.[45] In addition to these, several established and emerging techniques are available for measurement of peripheral vascular reactivity. These include: a) flow-mediated dilatation (FMD), b) changes in pulse wave velocity between the brachial and radial artery, c) hyperemic shear stress, d) reactive hyperemic flow, e) reactive hyperemia index (RHI) assessed by fingertip arterial tonometry, f) fingertip temperature rebound (TR), and g) skin reactive hyperemia.[46] Recently, measurement of NO via plasma nitrite concentration has also been utilized as a novel and more direct marker of endothelial dysfunction. A detailed description of all of these procedures is beyond the scope of this dissertation. However, three methods, namely the FMD of the brachial artery, reactive hyperemia index obtained by an EndoPAT tonometer and plasma nitrite are the focus of this dissertation and are discussed below in detail.

Methods of Measurement of Endothelial Function

(A) Flow-mediated dilation (FMD)

The underlying principle in the measurement of FMD is the production of NO derived from endothelial cells in response to a shear stress stimulus produced by either upper arm or forearm cuff occlusion of blood vessel. The blood vessel responds to this shear stress by regulating its tone, by dilating and increasing blood flow through its lumen. This phenomenon, known as flow-mediated dilation (FMD), is expressed as the percent change in blood vessel diameter after cuff release relative to its baseline and it is used as an index of endothelial function. Lower FMD signifies worse endothelial function. In addition to percent FMD, baseline vessel diameter and absolute change in vessel diameter can also be used as indices of endothelial function. This method was originally described by Celermajer et al[47] in the 1990's and it has been extensively used in cardiovascular research. Corretti et al provide detailed guidelines for the use of this procedure and careful review of its limitations and strengths.[48] Although FMD can be studied in radial, axillary and superficial femoral arteries, brachial artery is the most commonly used site due to its optimal size. The procedure uses a high-resolution ultrasound system equipped with vascular software for two-dimensional (2D) imaging, color and spectral doppler, an internal electrocardiogram (ECG) monitor and a high-frequency vascular transducer. After acquisition of a baseline resting image of the brachial artery and measurement of blood flow, arterial occlusion and ischemia are created by inflating a sphygmomanometric cuff to at least 50 mmHg above systolic blood pressure for a period of five minutes. Subsequent images are captured at 2 minutes during occlusion and serially up to 2 minutes after cuff deflation for measurement of brachial artery diameter

(See Appendix for Methods of Data Collection). Specialized vascular software can then be applied to read the captured images and obtain measurements of the changes in the brachial artery diameter.

There are several indications for measurement of FMD: (1) risk stratification for cardiovascular events i.e. to obtain prognostic information about cardiac events, (2) risk stratification in patients with chest pain or angina and to classify patients according to the extent of coronary artery disease, (3) risk stratification for postoperative cardiovascular events in patients undergoing vascular surgery, (4) evaluation of new therapies on endothelial function.[45] This technique has been shown to be highly reproducible under controlled conditions.[49, 50] Due to its non-invasive nature and high reproducibility, investigators have used this technique as an indicator of endothelial dysfunction to demonstrate the effects of air pollutants in controlled exposure studies.[37-39, 51] However, a few limitations of this procedure need to be acknowledged. First, it requires extensive sonographer training and use of high-resolution ultrasound equipment. Second, the analysis of images is operator-dependent, costly and time-consuming. Significant intra-observer and inter-observer variability may exist in the analysis of images requiring periodic assessment. Third, rigorous attention to protocol standardization, training and ongoing quality assurance is critical for acquisition of valid data. Given these limitations, assessment of endothelial function using an EndoPAT tonometer, as discussed below, is emerging as a substitute to FMD.

(B) EndoPAT tonometer

The Endo-PAT2000 is a non-invasive computer-based device (Itamar medical, Caesarea, Israel), intended for use as a diagnostic aid in the detection of coronary artery endothelial dysfunction using a reactive hyperemia procedure, identical to that used with FMD. The device has been approved by the United States Food and Drug Administration (USFDA) since 2004 and it is currently being incorporated into several large clinical studies conducted in the United States as well as other parts of the world. It is based on the use of Peripheral Arterial Tone (PAT) Technology and it attempts to measure post-ischemic arterial responsiveness to reactive hyperemia via measurement of blood volume. The procedure for acquisition of PAT data and clinical setting requirements for conducting EndoPAT studies are described in detail in the Appendix of this dissertation.

The use of the PAT technology was first published by Schnall et al in the year 1999 using finger plethysmographs in 42 patients with obstructive sleep apnea syndrome.[52] They observed profound, transient vasoconstriction and tachycardia with each apneic event in these patients. Subsequently, Rozansky et al compared pulse wave amplitude (PWA) responses to treadmill exercise in 50 normal volunteers and 57 patients with atherosclerotic coronary artery disease (CAD). [53] They demonstrated that patients with CAD manifested attenuation of PWA that was consistent with vasoconstriction during the early phase of exercise. The findings of this particular study spearheaded the use of the PAT device in cardiovascular research.

Several studies have demonstrated the utility of the PAT device as a diagnostic tool among patients with CAD. [53-60] Specifically, these studies have explored the utility of the device to address the following: (1) to identify individuals with abnormal coronary endothelial function, (2) to test the diagnostic capability of the device by

indentifying changes in PAT responses during mental stress or exercise, (3) to evaluate the utility of the device in identifying individuals with established diagnosis of CAD in an outpatient clinic setting, (4) to determine the relationship between PAT hyperemia and other established methods for measurement of endothelial function such as FMD, (5) lastly, to study the relationship between PAT hyperemia and cardiovascular risk factors (CRF).

Kuvin et al examined the relationship between number of CRF and PAT hyperemia response in two studies.[59, 60] These studies included adults with or without chest pain who underwent evaluation for the presence of CAD. The presence of the following CRF was assessed: male sex; hypertension, hyperlipidemia, diabetes mellitus, family history of CAD, postmenopausal status, and smoking. They observed that the PAT hyperemia ratio correlated with the number of CRF. They also reported that as the number of CRF increased, the PAT hyperemia ratio progressively decreased, indicating endothelial dysfunction. Furthermore, Hamburg et al (2008) examined this relationship in the Framingham Heart Study Third Generation Cohort in a community-based observational study. [61] They found certain risk factors to be inversely associated with the PAT ratio such as male sex, body mass index, ratio of total to HDL cholesterol, diabetes mellitus, smoking, and lipid-lowering treatment. Furthermore, investigators have reported a significant positive correlation between FMD and PAT hyperemia.[46, 59, 62] The estimated correlation coefficients ranged from 0.47 to 0.67 indicating a meaningful relationship between these two methods of measurement. The results obtained from these studies not only provide evidence supporting the utility of the device for conducting research in cardiovascular disease, but also suggest that the EndoPAT measurements may be an

attractive and simpler substitute for established methods of measurement of endothelial function. The utility of the PAT device has also been established in settings where short-term interventions or treatments have been shown to produce potential beneficial or worsening effects on endothelial function [54, 63-69] For instance, Bonetti et al examined the effect of enhanced external counterpulsation (EECP) on endothelial function among patients with established ischemic CAD.[54] The results showed an acute and significant increase in average PAT hyperemia ratio on all 3 EECP days following a one-hour treatment indicating transient improvement of endothelial function. In the field of environmental science, the PAT device has been employed to investigate the role of exposure to air pollution in causing endothelial dysfunction by Brauner et al.[63-64] Recently, they conducted two randomized cross-over studies in two different subject populations to investigate the effects of controlled exposure to particle-filtered and non-filtered air on endothelial function. While one of them showed a significant improvement in the PAT ratio following an exposure to filtered air, the other study failed to detect any differences.

Although evidence clearly suggests that the PAT device can be employed in different populations and different settings, one may come across a few challenges in acquisition of accurate and meaningful data using the device. Most importantly, there is lack of substantial evidence regarding the reproducibility of PAT measurements obtained a few hours apart on the same day as well as separate days. Prior to the design of studies that involve repeated PAT measurements, it is essential to investigate if such measurements are reproducible prior to the employment of the device in examining the acute and hyperacute (within a few hours) effects of air pollutants on endothelial

function. In addition, it is also crucial to examine the effects of mental stress on PAT measurements as a part of the validation process of the EndoPAT technology as commonly used study designs to examine the effects of air pollutants involve stress-inducing study protocols, which in turn, may have an effect on the PAT measurements.

(C) Plasma Nitrite Assay

Nitric oxide (NO) is a soluble gas synthesized in various mammalian cells.[70, 71] Nitric oxide synthase (NOS) are the enzymes responsible for NO generation. To date, three distinct isoforms of this enzyme have been identified: neuronal NOS (type I), inducible NOS (type II), and endothelial NOS (type III).[70] NO is the product of the five-electron oxidation of one of the chemically equivalent guanidinonitrogens of L-arginine by the NOS enzymes.[72] The substrate used for its synthesis is an amino acid L-arginine.[73] The rate of NO synthesis is critically influenced by various cofactors, like tetrahydrobiopterin (BH_4), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), the presence of reduced thiols, the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) and substrate availability. Additionally, endothelial NOS activity is dependent on calmodulin (caM) and Ca^{2+}. The three isoforms vary substantially in cellular location, structure, regulation, and functions. The endothelial NOS is mostly membrane bound and formed only in endothelial cells. The neuronal NOS was identified in the cytosol of central and peripheral neurons.[74] Both isoforms are present in resting state of cells and upon stimulation, generate small amounts of NO instantaneously for short periods and the process is calcium dependent.[71] In contrast, the third isoform i.e. inducible NOS or iNOS, is not present at rest but instead circulating monocytes and airway epithelial cells, in case

of exposure to air pollutants[75], are required to be induced in order to produce the enzyme via messenger RNA activation.

NO has an important role to play in the regulation of coronary and systemic vasodilator tone. The key cardiovascular functions of NO are as follows: (1) NO contributes to basal coronary artery vasodilator tone and blood flow on humans, (2) NO contributes to regulation of vascular tone and growth in the systemic circulation, (3) NO is involved in systemic and cerebral hypoxic vasodilation, (4) NO is also involved in homeostasis of blood pressure and vascular function, and (5) NO inhibits platelet activation, platelet aggregation and fibrinolysis.[76, 77]

Given its functions, measurement of NO bioavailability may be a clinically useful test in identifying individuals with certain pathologic conditions such as aging, hypertension, hypercholesterolemia, smoking, diabetes and congestive heart failure as these conditions are associated with endothelial dysfunction and impaired NO bioavailability. However, a short half-life and rapid metabolism of NO pose difficulty in accurate measurement of NO in humans.[70] NO that diffuses into the vascular lumen undergoes rapid metabolism in plasma and in red blood cells (RBC's). In plasma, NO is rapidly oxidized in the presence of oxygen.[70] Nitrite is a primary oxidative metabolite and comprises a major intravascular storage pool of NO.[70] Alternatively, NO can rapidly react with superoxide anions to produce the oxidant peroxynitrite ($ONOO^-$) and with oxygen to produce reactive nitrogen oxide species such as dinitrogen trioxide.[70] Lauer et al tested nitrite, nitrate and total NOx levels among 24 healthy volunteers and reported that endothelial NOS stimulation with acetylcholine augmented venous nitrite levels by 71% and it also increased forearm blood flow by 4-fold. [78] However, these changes were not

observed in venous nitrate and total NOx levels. Therefore, they further concluded that plasma nitrite reflected endothelial NOS activity, rather than nitrate. There is evidence that 90% of circulating nitrite endogenously originated from the L-ariginine/NO pathway.[79] These findings support the use of plasma nitrite concentration as an acute biochemical marker of NO activity in humans.

It has been previously shown that reduced nitric oxide availability is a marker of endothelial dysfunction. Kleinbongard et al examined whether plasma nitrite levelsare reduced in human volunteers with endothelial dysfunction and if the decrease is correlated with the number of cardiovascular risk factors.[80] In the first cross-sectional study, they concluded that plasma nitrite levels decreased progressively with increasing number of cardiovascular risk factors and there was a significant correlation between plasma nitrite and age, mean arterial blood pressure, hypercholesterolemia and smoking. In the other part of the study, they concluded that decreasing levels of nitrite were found in patients with established endothelial dysfunction i.e. patients with cardiovascular disease. This study also confirmed two important findings. First, plasma nitrite levels were positively correlated with BAFMD. Second, the sensitivity of nitrite for the detection of endothelial dysfunction was calculated to be 64% with a specificity of 81%. Another study by Kehmeier et al reported lower whole blood nitrite levels among MI patients admitted to an intensive care unit where the investigators examined the ease of measurement of nitrite levels among critically ill patients and also demonstrated endothelial dysfunction in these patients.[81] Recently, it has been shown that plasma nitrite increased by roughly 52.5% following reactive hyperemia when blood samples were obtained prior to and 60 seconds following a five-minute occlusion of blood flow in the

upper arm in 15 healthy subjects.[82] This experiment opened a new avenue to utilize measurement of nitrite following a hyperemic stimulus as a marker of localized NO production. Even though this study could not demonstrate significant differences in plasma nitrite concentration between healthy volunteers and patients with cardiovascular disease, there is some evidence that plasma nitrite concentration increases following a hyperemic stimulus among healthy volunteers, but not in patients with endothelial dysfunction.[83] In addition to the hyperemic stimulus, graded exercise test was recently used by Allen et al (2009) to stimulate nitrite levels in 4 groups of patients with increasing severity of endothelial dysfunction.[84] These groups were as follows: (1) risk factors, but no vascular disease, (2) type II diabetes mellitus (DM) with no vascular disease, (3) diagnosed peripheral arterial disease (PAD), and (4) DM + PAD. Following the exercise test, plasma nitrite was shown to increase in the group with risk factors, no change in the DM group, and a decrease in the PAD and PAD + DM groups. This study demonstrated the utility of plasma nitrite measurement as a biochemical marker for differentiating patients with varying degrees of endothelial dysfunction.

Measurement of plasma nitrite has not been extensively used to study the effects of environmental exposures on endothelial function. However, this test can accurately reflect the bioavailability and activity of NO if appropriate techniques of blood sampling, processing and analysis are used. In this dissertation, the EndoPAT measurements and plasma nitrite levels were used as additional markers of endothelial dysfunction in addition to the BAFMD.

In summary, the overall goal of this dissertation was to first validate the EndoPAT technology by conducting experiments to examine its reproducibility and then

to examine the effects of air pollutants on three endothelial function markers, namely BAFMD, EndoPAT measurements and plasma nitrite concentration in a controlled exposure study setting as well as a 'real world' setting.

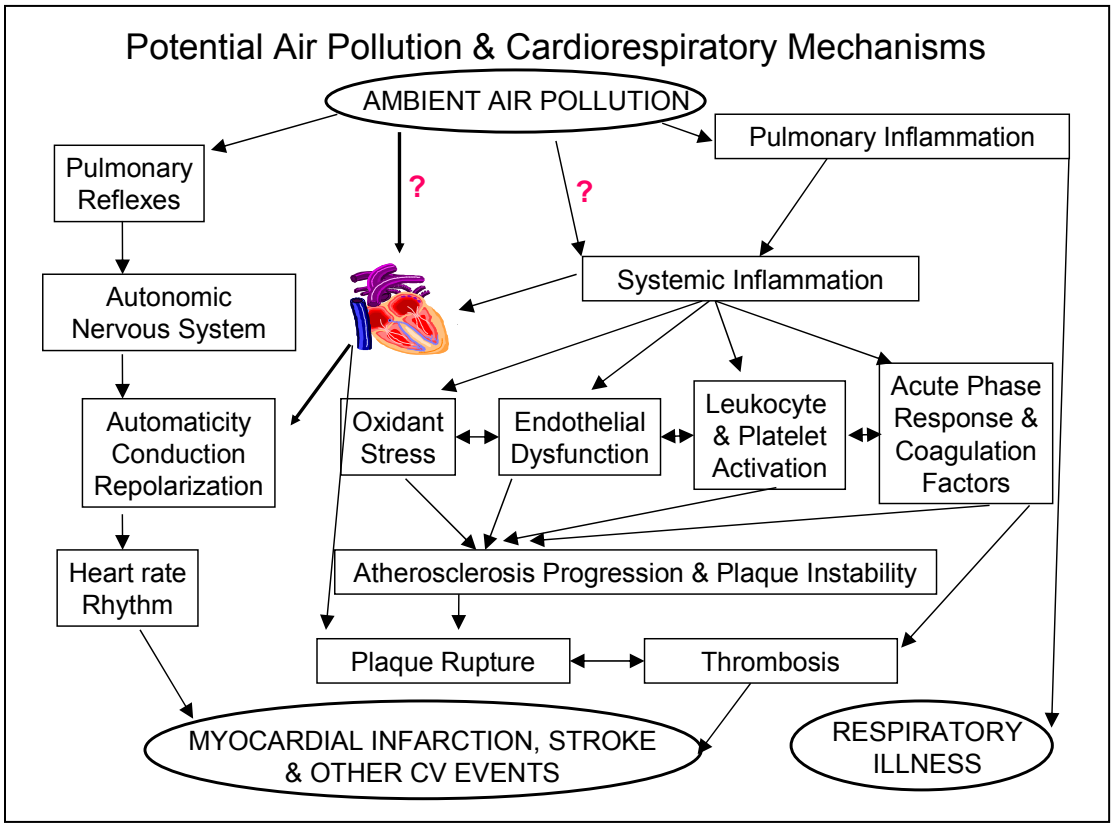
The specific aims of this dissertation are:

(1) To examine the intra-day reproducibility of PAT measurements (i.e. baseline pulse wave amplitude and PAT ratio) obtained 2.5 hours apart and the inter-day reproducibility of PAT measurements obtained 1 week apart at baseline, and (2) examine the effects of mental stress on EndoPAT measurements in a group of young and healthy volunteers in manuscript 1.

(2) To examine the acute changes in PAT measurements, plasma nitrite concentration and FMD of the brachial artery following a 2-hour exposure to fresh diesel exhaust (200 $\mu g/m^3$) and secondary organic aerosols (200 $\mu g/m^3$) in young and healthy volunteers in a controlled exposure study in manuscript 2.

(3) To examine the changes in PAT measurements and plasma nitrite concentration associated with increases in five ambient air pollutant concentrations, namely $PM_{2.5}$, carbon monoxide, sulphur dioxide, nitrogen dioxide and ozone in the preceding 7 days in manuscript 3.

Figure 1. Possible Mechanisms by which Air Pollutants may Cause Cardiovascular Illnesses



Adapted from Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M. Air Pollution and Cardiovascular Disease: A Statement for Healthcare Professionals From the Expert Panel on Population and Prevention Science of the American Heart Association. Circulation 2004; 109;2655-2671.

Table 1. Sources and Health Effects of Gaseous Air Pollutants

Pollutant	Type of Substance	Sources	Health Effects
Nitrogen oxides (NOx)	Reactive gases	Motor vehicle emissions, power plants	Airway inflammation and exacerbation of asthma, aggravation of existing heart disease
Carbon Monoxide (CO)	Colorless gas	Motor vehicle exhaust, industrial processes, residential wood burning	Reduction of oxygen delivery to tissues due to greater affinity for hemoglobin, individuals with preexisting cardiovascular disease are at higher risk for developing adverse effects of CO.
Sulphur dioxide (SO_2)	Colorless soluble gas with pungent odor	Electricity generation, fossil fuel combustion at power plants	Exacerbation of asthma, bronchoconstriction
Ozone (O_3)	Highly reactive colorless to bluish gas	Created by a chemical reaction between nitrogen oxides and volatile organic compounds	Aggravation of asthma, worsening of bronchitis, emphysema

Adapted from U.S.EPA website and Adapted from Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M. Air Pollution and Cardiovascular Disease: A Statement for Healthcare Professionals From the Expert Panel on Population and Prevention Science of the American Heart Association. Circulation 2004; 109;2655-2671.

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VALIDATION OF ENDOPAT TECHNOLOGY

PART ONE: INTRA AND INTER-DAY REPRODUCIBILITY OF ENDOPAT MEASUREMENTS

by

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ABSTRACT OF MANUSCRIPT 1 (PART 1) OF 3

INTRA AND INTER-DAY REPRODUCIBILITY OF ENDOPAT MEASUREMENTS

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ABSTRACT

Introduction: Substantial evidence regarding the reproducibility of EndoPAT (or PAT) measurements obtained on the same day as well as on separate days is lacking. Prior to the design of studies that involve repeated PAT measurements on young and healthy individuals, it is essential to investigate if such measurements are reproducible.

Methods: Two PAT measurements i.e. PAT ratio and pulse wave amplitude (PWA), obtained 2.5 hours apart from 10 young and healthy participants who underwent PAT testing alone in a pilot experiment were used to examine the intra-day reproducibility (Aim 1a). Sixty-three college students were enrolled to participate in an exposure study where subjects were randomly assigned to a 2-hour exposure to either two air pollutants or clean air (CA) in 3 exposure sessions conducted one week apart. Of these, the PAT measurements obtained before and after the exposure to CA from 48 subjects were used to examine the intra-day (Aim 1b). Up to 3 PAT measurements obtained at baseline prior to the exposure to air pollutants one week apart from 50 subjects were used to examine the inter-day reproducibility (Aim 2). To examine each aim, an intra-subject coefficient of variation (CV) and an intra-class correlation coefficient (ICC) were calculated.

Results: The mean PAT ratios showed a tendency towards an increase after repeat testing in both the pilot and CA exposure settings, with a significant increase in the latter setting.

The ICC's for the PAT ratio were low [Pilot study ICC (95% CI): -0.07 (-0.63, 0.55); CA exposure study ICC: 0.40 (0.14, 0.61)], indicating poor intra-day reproducibility. The PAT ratio obtained a week apart also exhibited a low inter-day reproducibility. The baseline PWA was reproducible when measured one week apart, but not 2.5 hours apart following an exposure to clean air in the CA exposure study.

Conclusions: Our study concluded that the PAT device does not produce reliable PAT ratio estimates when tests are repeated 2.5 hours apart and one week apart in young and healthy population. Therefore, prior to designing studies to examine the effects of interventions on endothelial function using this technique in young and healthy population, investigators should be cautious while interpreting the effects of interventions on endothelial function based on a PAT ratio alone. Instead of examining a change from baseline to post-intervention in the PAT ratio, only post-intervention PAT ratios may be utilized to examine the effect of an intervention. Further studies are required to examine if the change in the PAT ratios within a short time interval is reproducible on different days.

INTRA AND INTER-DAY REPRODUCIBILITY OF ENDOPAT MEASUREMENTS

Introduction

Peripheral arterial tonometry (PAT) is a novel non-invasive method of evaluating endothelial function. It uses an EndoPAT (or PAT) tonometer device (Itamar medical, Caesarea, Israel) to measure post-ischemic arterial responsiveness to reactive hyperemia induced by upper arm blood flow occlusion. The utility of the PAT device has been established in various populations and in testing the effects of short-term interventions on endothelial function.[1-11] In order to capture the response of an acute intervention such as a dietary or medication change on endothelial function, it is usually necessary to obtain a pre-intervention and multiple post-intervention measurements using the device within a short interval of time on the same day as well as on separate days. Thus, prior to designing studies involving repeated measurements with the device, it is indicated to investigate whether such measurements are reproducible.

A few studies have examined the inter-day reproducibility of PAT measurements, providing inconsistent evidence. [1, 9, 12-16] One study reported minimal day-to-day variability in the measurements only based on a Bland-Altman plot when the measurements were obtained on two consecutive days in 28 young volunteers.[9] Another study conducted in adolescents with type I diabetes reported a coefficient of variation (CV) of 14.8% when measurements were repeated 4 weeks after the initial testing.[14] More recently, four studies conducted on healthy adults reported an intra-class correlation coefficient (ICC) ranging from 0.56 to 0.78 as a measure of reproducibility on PAT studies performed at least a day apart, but separated by no more than 7 days except one.[1,

[12, 13, 16] While these studies suggested a moderate to good inter-day reproducibility of the PAT measurements, Liu et al (2009) provided contrasting findings.[15] This study reported a low inter-day reproducibility of PAT measurements in 10 healthy volunteers when the tests were repeated on 3 consecutive days at the same time of day based on low ICC's.[15]

There is also limited evidence regarding the reproducibility of PAT measurements obtained a few hours apart on the same day. Crandall et al (2009) reported a CV of 15.2% for test-retest repeatability among healthy subjects when measurements were repeated two hours apart.[3] Liu et al reported that PAT ratios showed a significant increase from measurement 1 to measurement 6 when tests were repeated every half an hour.[15] However, the same study concluded that repetitive measurements had no carryover effect on the PAT ratio at 1 and 2 hour intervals, and intra-subject CV was similar to that observed in studies of flow mediated dilation using brachial artery ultrasound scanning.

Previous studies have examined the reproducibility of PAT tests in a setting where tests were conducted in healthy volunteers and participants underwent no other interventions, but PAT testing alone. However, the reproducibility of this technique has not been examined in study designs that concurrently involve a battery of invasive procedures such as venipuncture and collection of biological samples in a controlled environment. There is some evidence that venipuncture procedure which leads to injury to blood vessel and activation of platelets can cause vasoconstriction and increased vascular tone via release of various clotting factors such as thromboxaneA2 [17], platelet activating factor [18, 19] and serotonin.[20] While these findings have been reported using animal models, Ward et al (1983) have reported that exposure to stressors such as

venipuncture significantly increased the release of epinephrine and norepinephrine in humans, which in turn may be responsible for increased vascular tone.[21] Since the study designs aimed at examining endothelial dysfunction as an acute consequence of exposure to air pollutants usually involve venipuncture and other invasive procedures[22-24], it is imperative to investigate the reproducibility of repeated PAT measurements in such study designs. To address these questions, we sought to examine: (1) the intra-day reproducibility of PAT measurements obtained 2.5 hours apart in two settings: (a) the one where participants underwent no other interventions, but PAT testing alone in a pilot study; (b) the other where participants underwent the interventions listed above as well as an exposure to clean air in a controlled environmental facility as part of a controlled exposure study; and (2) the inter-day reproducibility of PAT measurements obtained 1 week apart at baseline in a group of young and healthy volunteers.

Materials and Methods

Data Sources

Data to examine Aim 1a were obtained from a pilot study. Ten healthy and non-smoking subjects between the ages of 18-45 years were recruited from the Rutgers University community using advertisement flyers. The study was conducted at the Clinical Center of the Environmental and Occupational Health Sciences Institute (EOHSI) located in Piscataway, New Jersey. The pilot study was approved by the University of Medicine and Dentistry of New Jersey (UMDNJ)-Robert Wood Johnson Medical School Institutional Review Board.

Data to examine Aim 1b and Aim 2 were obtained from a controlled exposure study. A double-blind crossover experiment using three different controlled exposure conditions was conducted at the EOHSI to examine the effects of a 2-hour exposure to two kinds of air pollution particles, namely diesel exhaust (DE) and secondary organic aerosol (SOA) on platelet activation markers and endothelial function in a group of 63 young and healthy volunteers. Data on both outcomes were also collected following a 2-hour exposure to filtered clean air (CA) for comparison. This study was approved by the UMDNJ-Robert Wood Johnson Medical School Institutional Review Board.

Subject Recruitment and Screening

Out of the ten subjects enrolled in the pilot study, 5 subjects were staff members involved with research activities at the EOHSI and the other 5 subjects were students at the Rutgers University. All subjects were between 18-45 years and were recruited in the months of August and September in the year 2008. When a potential candidate contacted research staff, he/she was screened to ascertain his/her eligibility to participate in the study using a standard telephone questionnaire. Subjects were excluded if they were smokers, indicated presence of cardiovascular disease, cancer, stroke, hypertension, asthma, neurological disease, kidney and liver disease. Additional exclusions were: pregnant or breastfeeding females, individuals currently taking daily anti-hypertensive medications or any other vaso-active medications, and individuals with known allergies to latex.

Sixty-three healthy and non-smoking subjects between the ages of 18-30 years were recruited to take part in the controlled exposure study from the Rutgers University community using advertisement flyers. All subjects were recruited over a period of 4 years from December 2005 and April 2009. Potential candidates who were interested in participation were screened by the research staff using a standard telephone questionnaire to determine their eligibility for participation. In addition to the exclusion criteria listed for the pilot study, candidates were excluded if they were prescribed to take any aspirin containing medications on a daily basis to avoid the effect of such medications on platelet activation markers.

Clinical Evaluation of Subjects

No further clinical evaluation was conducted for the pilot study participants. However, the candidates deemed eligible at the initial telephone screening for the exposure study were scheduled to complete a history and physical examination under a study physician's supervision. The physical examinations were conducted at the EOHSI Clinical Center. All subjects provided a written informed consent at this appointment. The purpose of the physical examination was to rule out presence of any medical conditions that would preclude study participation. The appointment included a vital examination, routine blood chemistries (complete blood count with differential and platelet count), measurement of blood pressure, an electrocardiogram, lung function test and a medical examination by a physician. In addition, subjects were asked to complete a medical questionnaire that asked history of medical illnesses and prior as well as current use of medications. No clinically significant abnormalities were allowed to be present. The following inclusion

and exclusion criteria were used to ascertain eligibility: (a) Inclusion criteria: age between 18-30 years**,** non-smokers, (b) Exclusion criteria: neurologic disease**,** stroke, cardiovascular disease**,** pulmonary disease including asthma**,** gastrointestinal disorders, known endocrine disease**,** major psychiatric conditions, pregnant or lactating women, and female subjects using any form of hormonal contraception such as oral contraceptive pills or patch. Medical records obtained from the physical examination were evaluated by the study physician to ascertain eligibility. Based on the physician's review, subjects were either invited to participate in the study or declined participation due to medical reasons. In addition to the clinical evaluation, subjects were familiarized with a controlled environmental facility (CEF) located on the third floor of the EOHSI where the exposure sessions were conducted. The research staff briefly explained experimental procedures at this appointment.

Study Protocols

Pilot study subjects were scheduled for one session lasting for approximately 4 hours from 8:00 A.M. to 12:00 P.M. Subjects were asked to arrive following an overnight fast beginning at 12:00 P.M. the night before the day of testing. They were asked to avoid consumption of alcoholic and/or caffeinated beverages and any vasoactive medications on the day of testing. Subjects reported to the EOHSI Clinical Center at 8:00 A.M. Upon their arrival, the research staff obtained a written informed consent. The research staff also performed a check-in to ascertain if subjects have followed the above-mentioned instructions. A pregnancy test was administered to women**.** A baseline (or first) PAT test was conducted using a standardized protocol by an experienced PAT technician (See

Appendix of the dissertation for details on methods of data collection). The PAT tests were performed in a dark, noise, and temperature controlled room of the Clinical Center. Following the baseline PAT test, subjects were asked to relax or read for a period of 2.5 hours. Out of the 10, 7 subjects left the facility and relaxed at a location other than the Clinical Center, for example – work desk or a computer lab. However, they were instructed to refrain from any physical activity during this period. Three subjects stayed at the Clinical Center during the rest period. A repeat (or second) PAT test was conducted at the end of the rest period. Subjects remained fasting until the repeat PAT test was completed.

Once the candidates for the exposure study were medically cleared by the study physician, they were scheduled for three exposure sessions conducted in the morning and three follow-up visits conducted in the afternoon on the same day. In most cases, the actual study participation was completed within two months of subjects' physical exam appointment. Each subject underwent testing one day per week over 3 consecutive weeks on the same day of the week. For the most part, all visits were conducted at least one week apart. Subjects were randomly assigned to receive three exposure conditions, namely, DE, SOA and CA. The research staff who conducted the experiment and subjects were kept blind to the order of exposure. All outcomes including assessment of endothelial function were evaluated at baseline, i.e. before entering the CEF and immediately following a 2-hour exposure to DE, SOA or CA. Subjects were asked to arrive following an overnight fast beginning at 12:00 midnight the day before testing. They were instructed to abstain from: (1) any caffeinated and/or alcoholic beverages and vigorous exercise 12 hours prior to the testing, and (2) aspirin containing

medications 2 weeks prior to the testing to avoid their effect on platelet activation markers (one of the endpoints described elsewhere). On the day of each experimental session, subjects reported to the Clinical Center at 8:00 A.M. and the research staff performed a check-in to ascertain that the above conditions have been met. A pregnancy test was administered to women. A pre-exposure blood sample was collected into vacuum tubes and aliquotted for measurement of plasma nitrite and other biomarkers of inflammation. Then endothelial function was measured using two methods simultaneously, namely, the EndoPAT tonometer and brachial artery ultrasound scanning (See Appendix for methods of data collection), approximately between 8:30 A.M and 9:00 A.M. The PAT test conducted at this time point was referred to as a baseline or pre-exposure PAT test. Subjects were then taken to the CEF and seated comfortably in a chair. Subjects were exposed for approximately 2 hours to either DE (200 $\mu g/m^3$), SOA (200 $\mu g/m^3$), or filtered CA in a random order. At the end of the 2-hour exposure session, another blood sample was obtained for measurement of the same parameters mentioned above. Following the venipuncture, the endothelial function measurement using both methods was repeated approximately between 12:00 P.M. and 12.30 P.M, on an average 2.5 hours after the baseline measurement. The PAT test conducted at this time point was referred to as a post-exposure PAT test. Of the 63 subjects enrolled, pre and post-exposure PAT measurements on the CA day were available on 48 subjects and were used to examine Aim 1b. The reasons for availability of complete PAT data on a reduced number of subjects compared to all those who were enrolled in the study (N=63) have been explained in the manuscript two of this dissertation (refer to pages 116-117 under Availability of Data). Data collected on PAT measurements one week apart at baseline

(prior to the exposure to air pollutant particles or CA) were used to examine Aim 2. Depending upon the number of visits completed by each subject, at least one and up to three baseline PAT measurements were available from each subject. Data from 50 subjects with at least two and up to three baseline PAT measurements were available for analysis specified under Aim 2.

Study Variables

A detailed description of the methods of data collection using the EndoPAT device is provided in the appendix. The main study variables were as follows: (1) baseline pulse wave amplitude (PWA) for the control arm and the occlusion arm, and (2) PAT ratio.

Clean Air Exposure Condition

For the controlled exposure study, exposure sessions were conducted in the CEF. The CEF located on the third floor of the EOHSI is a large stainless steel chamber in which conditions of temperature and humidity are controlled. The chamber dimension is 7.3 ft. high by 13.5 ft. wide by 9 ft. deep for a volume of 887 cubic ft. (or 25 m^3). The chamber is interfaced to maintain constant environmental (exposure) conditions for human health effects studies. It has tables, chairs, a personal computer, and a lavatory for subject use during experiments. Ambient air was drawn into the CEF through coarse particle air filters and High-Efficiency Particulate Air (or HEPA) filters and filtered across activated carbon to remove ozone and organics. Mean, minimum, and maximum pollutant concentrations for clean air exposure session during the study have been provided in Table 3 of manuscript two of the dissertation.

Statistical Analyses

Intra-day reproducibility: Demographic characteristics of the subjects such as age, gender, and race were reported for the pilot study and the controlled exposure study participants separately. Baseline clinical characteristics such as weight, body mass index and lipid profile measurements were reported for the exposure study participants only. Descriptive statistics of the baseline (or first) and repeat (or second) PAT test study variables such as means and standard deviations were reported. A paired t-test was performed to determine if the two PAT test variables were significantly different. Two measures of reproducibility, namely (a) intra-subject coefficient of variation (CV), and (b) intra-class correlation coefficient (ICC) were calculated as follows:

(a) An individual CV was calculated using the following formula-

$CV_{Individual}$ = (SD of two measurements/Mean of two measurements) X 100. A mean CV and its 95% confidence interval (CI) were calculated, (b) ICC's and their 95% CI's were computed using SPSS version 18.0 (SPSS, Inc., Chicago IL). A within-subjects analysis of variance (ANOVA) one-way random effects model was fit where two measurements of the PAT test were obtained on a sample of 'n' subjects (Pilot study n:10, Exposure Study n: 48). A sensitivity analyses was performed for the CA exposure setting to explore if the order of the visit (first, second, or third exposure session) had any effect on the measures of reproducibility of PAT measurements.

Inter-day reproducibility: Descriptive statistics such as mean and standard deviation of the PAT measurements obtained 1 week apart at baseline prior to the exposure were reported. Due to the varying number of visits completed by subjects (either two or three),

mean PAT measurements on each visit were reported on two overlapping groups of subjects as follows: (1) Group 1: included data from 36 subjects on whom complete data on three visits were available (N=36 subjects, 3 PAT tests per subject, Total 108 PAT tests), (2) Group 2: included data from 50 subjects on whom complete data on at least two visits were available (N=50 subjects, 2 PAT tests per subject, Total 100 PAT tests). Repeated measures ANOVA and a paired t-test were performed to examine if the mean PAT measurements were significantly different in group 1 and 2, respectively. A mean intra-subject CV and ICC's were calculated by using the same method described above. All analyses (except the computation of ICC) were conducted using the SAS programming language version 9.3 (SAS Institute, Inc, Cary, NC).

Results

Baseline Characteristics of Study Subjects

Demographic and baseline clinical characteristics of subjects from the pilot and exposure studies are presented in Table 1. The mean age of the pilot study subjects was 28.7 years (Standard deviation=8.2). Nine out of the 10 enrolled subjects were white men. Mean systolic and diastolic blood pressure measurements were higher in the pilot study participants as compared to the exposure study participants. Information on baseline clinical characteristics other than blood pressure was not collected.

The mean age of the subjects who participated in the CA exposure study was 21.3 years (Standard deviation=3.1). Predominantly, this study population was also comprised of white men. Body mass index and other clinical parameters were in the

normal range. Clinical characteristics of the subjects from the inter-day experiment were similar to the subjects from the CA exposure study as shown in Table 1.

Aims 1a and 1b: Intra-day Reproducibility of PAT Measurements

Pre-Post Changes in the Mean PAT measurements

Changes in the mean PAT measurements from baseline (first test) to repeat testing in the pilot study and pre to post CA for the CA exposure study are shown in Table 2. In the pilot study, 4 of the 10 subjects showed an increase in the PAT ratio whereas 6 subjects showed a decrease in the PAT ratio (Figure 1).

The mean PAT ratio from the baseline to repeat testing showed a trend towards an increase. When the differences in the PAT ratio were tested using a paired t-test, it showed a statistically non-significant trend towards an increase in the PAT ratio (1.65 ± 0.3 vs. 1.82 ± 0.6, $p=0.45$).

In the pilot study, a decrease in the mean PWA from the baseline to the repeat test was observed in 7 and 8 subjects in the occlusion and the control arm, respectively (Figure 2). When the differences in the PWA were tested using a paired t-test, the PWA for both arms showed a non-significant decrease from baseline (first) to the repeat test ($p=0.05$ and 0.28 for control and occlusion arms, respectively) (Table 2).

In the CA exposure study, 31 of the 48 subjects showed an increase in the PAT ratio whereas 17 subjects showed a decrease in the PAT ratio from pre to post CA testing (Figure 3). The mean PAT ratio from pre to post CA exposure showed a trend towards an increase. When the differences in the PAT ratio were tested using a paired t-test, it showed a significant increase following the exposure to CA (1.65 ± 0.4 vs. $1.86 \pm$

0.5, p=0.005) (Table 2). A decrease in the PWA from pre to post CA test was observed in 44 and 43 subjects (out of 48) in the occlusion and the control arm respectively (Figure 4). When the differences in the PWA were tested using a paired t-test, the mean PWA showed a significant decrease from pre to post CA exposure in both arms ($p < 0.0001$ for both arms) (Table 2).

Comparison of PAT Measurements from the Pilot and the CA exposure Studies

Table 3 shows two comparisons: (1) baseline PAT test from the pilot study versus the pre-CA PAT test from the CA exposure study, and (2) repeat Pat test from the pilot study versus post-CA PAT test from the CA exposure study. The mean PAT ratio obtained at baseline (i.e. prior to CA exposure) from the CA exposure study was not significantly different from the PAT ratio of the first PAT test from the pilot study (1.65 ± 0.4 vs. 1.65 ± 0.3, p=0.99). However, the mean PWA for both arms at baseline from the CA exposure study was significantly lower than the PWA of the first PAT test from the pilot study (Control arm: 605.5 ± 428.8 vs. 945.6 ± 405.0, p=0.03, Occlusion arm: 616.5 ± 404.8 vs. 941.0 ± 343.9, p=0.02). Similarly, the mean PAT ratio obtained after the CA exposure from the CA exposure study was not significantly different from the mean PAT ratio of the repeat PAT test from the pilot study (1.86 ± 0.5 vs. 1.82 ± 0.6, p=0.86). However, the PWA for both arms at post CA exposure from the CA exposure study was significantly lower than the PWA of the repeat PAT test from the pilot study (Control arm: 187.5 ± 160.3 vs. 752.7 ± 406.9, $p<0.0001$, Occlusion arm: 192.8 ± 157.8 vs. 818.6 ± 404.1, $p<0.0001$).

Intra-day Reproducibility of PAT Measurements

Table 4 shows the measures of reproducibility for PAT measurements for the pilot and the CA exposure studies.

PAT Ratio: The mean intra-subject CV for the CA exposure study was lower as compared to the intra-subject CV from the pilot study [16.7% (95% CI:13.5%, 19.9%) vs. 19.8% (95% CI:8.3%, 31.2%)] but the CV's were not statistically different (p=0.47). The ICC's for both studies were either negative or low indicating that the within-subject variability for the pilot study was much greater compared to the between-subject variability.

PWA: The CV's from the CA exposure study were much greater as compared to the pilot study, likely reflecting a large variation in PWA's from pre to post CA testing (70.4% for the occlusion arm in the CA exposure study vs. 30.7% for the occlusion arm in the pilot study). The CV for the PWA of the pilot study was also greater compared to the CV's of the PAT ratio. These high values reflect the changes in the PWA from the first to the repeat testing. The ICC's for PWA's ranged from 0.58 to 0.70 in the pilot study indicating moderate reproducibility. Contrary to that, the ICC's were negative for the CA exposure study indicating a poor reproducibility. Overall, the PAT ratios were not reproducible in both settings (i.e. pilot and CA exposure study) and the PWA was reproducible in the pilot setting, but not in the CA exposure setting.

Effect of Order of Visit on Pre-Post Changes in the Mean PAT measurements in CA Exposure Study

The mean changes in the PAT measurements were examined by the order of visit where CA was the subject's first, second or third exposure session in the CA exposure study (Table 5). The PAT ratio did not show any changes from pre to post CA when CA was the subject's first exposure session. However, the PAT ratio showed a tendency towards an increase from pre to post CA when CA was subject's second visit (1.66 ± 0.5 vs. 1.82 ± 0.5, p=0.26) and the mean Pat ratio showed a significant increase from pre to post CA when CA was subject's third visit (1.67 ± 0.5 vs. 2.18 ± 0.6, p=0.002). When the differences in the PWA from pre to post CA were tested using a paired t-test for each visit, the PWA showed a significant decrease from pre to post CA exposure in both arms independent of the order of visit. The PWA was the lowest at the pre-CA PAT test when it was the subject's first exposure session, thereafter, the PWA of the pre-CA PAT test increased with the order of the visit. For instance, the mean (± Standard deviation) PWA for control arm when CA was the first visit was 431.8 ± 399.6 compared to 718.6 ± 362.9 when CA was the second visit and 721.1 ± 496.5 when CA was the third visit. Measures of reproducibility by order of visit are displayed in Table 6. The intra-subject CV for the PAT ratio was the lowest when CA was subject's first visit with successive increase in the CV from visit 1 to visit 3 (13%, 18.6% and 19.9% respectively). The ICC for the PAT ratio was the highest when CA was subject's first visit as opposed to when it was second or third visit. A low CV and a high ICC on the first visit indicated that the mean PAT ratios changed the least when CA was the subject's first visit as opposed to second or third visit. The intra-subject CV's and the ICC's for PWA were high and low or negative respectively, indicating poor reproducibility. This finding indicated that regardless of the order of the visit, the PWA showed a poor reproducibility in the CA exposure study.

Aim 2: Inter-day Reproducibility of PAT Measurements

Changes in the Mean PAT measurements by Visit

Figure 5 displays the variation in the PAT ratios by visit obtained from 36 subjects (group 1) on whom complete data on all three visits were available. In this group, the mean PAT ratio showed no change from visit 1 to 2, with an increase in the third visit (Table 7). However, the mean (± Standard deviation) PAT ratios from visit 1 to visit 3 were not significantly different from one another (Visit 1: 1.64 ± 0.4; Visit 2: 1.66 ± 0.5; Visit 3: 1.75 ± 0.5; p=0.53). Figure 6 displays the variation in the PWA of the occlusion arm by visit obtained from 36 subjects. The Mean PWA for both arms showed a tendency towards an increase from visit 1 to 3, however, the mean PWA from visit 1 to 3 were not significantly different (p=0.32 for control arm; p=0.16 for occlusion arm) (Table 7).

Figures 7 and 8 display the variation in the PAT ratios and PWA of the occlusion arm by visit obtained from 50 subjects (group 2) on whom complete data on only 2 visits were available. In this group, the mean PAT ratios showed no change from visit 1 to 2 (p=0.86) (Table 7). The Mean PWA for both arms showed a tendency towards an increase from visit 1 to 2, however, the mean PWA from visit 1 and 2 were not significantly different (p=0.09 for control arm; p=0.19 for occlusion arm) (Table 7).

Inter-day Reproducibility of PAT Measurements

Table 8 shows measures of inter-day reproducibility for PAT measurements.

PAT Ratio: The CV [17.1% (95% CI: 13.7%, 20.6%)] was low. However, the ICC of 0.27 (95% CI: 0.07, 0.49) based on the subjects from group 1 indicated a low

reproducibility. The ICC calculated based on the data from subjects in group 2 was comparatively higher than the ICC from group 1 subjects [0.43 (95%CI: 0.18, 0.63)]. As part of sensitivity analyses, ICC's were also calculated on residuals of the PAT ratios. However, the estimates didn't change after removing the effect of visit [0.28 (95%CI: 0.07, 0.50)], indicating that the order of visit had no effect on the reproducibility of the PAT ratio.

PWA: An intra-subject CV was 40.3% and 40.4% for the control and occlusion arm respectively. For the 36 subjects in group 1, an ICC of 0.70 and 0.60 for the control and the occlusion arm, respectively was observed indicating moderate reproducibility. ICC's based on the data from group 2 subjects were comparable with the ICC's from group 1 subjects.

Discussion

In a study conducted to examine the intra-day and inter-day reproducibility of PAT ratio and pulse wave amplitude, we found that: (1) the mean PAT ratios showed a tendency towards an increase after repeat testing within 2.5 hours in both the pilot and CA exposure settings, with a significant increase in the latter setting attributed to larger sample size, (2) while the CV's for the PAT ratio were low and in the range previously reported, either a negative or low ICC's for the PAT ratio in both study settings indicated that two PAT measurements obtained 2.5 hours apart from a single subject vary almost as much as any randomly chosen PAT ratio measurements from two different subjects, and (3) the PWA had a tendency to decrease after repeat testing in both settings, with a significant decrease observed following the exposure to CA. The PWA showed a

moderate reproducibility in the pilot study, but a poor reproducibility in the CA exposure study, (4) the PWA of the pre-CA PAT test in the CA exposure study was significantly lower than the PWA of the first PAT test of the pilot study, especially when CA was the subject's first visit. This finding suggested that the exposure study protocol, which involved an exposure to different stressors such as venipuncture and anticipation of being exposed to air pollutants, itself, had a significant lowering effect on the PWA. (5) although the intra-subject CV was low and in the range previously reported, a low (0.27 to 0.43) indicated a low inter-day reproducibility of the PAT ratio whereas the PWA showed a moderate inter-day reproducibility.

The mean PAT ratios showed a significant increase following the exposure to clean air, suggesting either possible carry-over effect on endothelium from repetitive hyperemia or a 'real' improvement in endothelial function. Although there is prior evidence that exposure to filtered clean air improves endothelial function as reported by a study from Denmark where adult volunteers were exposed to particle-filtered air in their homes for 48 hours prior to PAT testing compared to non-filtered air [23], it is unlikely that the increase observed in the PAT ratio in our study implies an actual improvement in endothelial function. This is due to the fact that a similar increase in the PAT ratio was also observed following an exposure to air pollutants such as diesel exhaust when PAT tests were repeated following the exposure to particles as discussed in manuscript two of this dissertation.

While no studies involving PAT testing as a primary outcome have reported the changes in baseline PWA, (a unitless variable automatically provided by the device), we observed two novel findings with respect to the changes in PWA in both the

pilot and CA exposure studies: (1) the baseline (pre-CA) PWA for the CA exposure study was significantly lower compared to the first PAT test PWA for the pilot study. The decrease in the PWA can be interpreted as an increase in peripheral arterial tone, or simply vasoconstriction. The PWA measurements from these two settings only differed on one point that the CA exposure study involved venipuncture immediately prior to the PAT testing unlike the pilot study design. This procedure itself may lead to vasoconstriction as exhibited by the decrease in the PWA. We also speculate that the subjects from the CA exposure study may have been anxious in anticipation of being in the exposure chamber and an exposure to air pollutants as they were unaware of the order of exposure involved and this anxiety and/or stress perhaps contributed to the observed decrease in the PWA, (2) we also observed a significant decrease in the PWA following the exposure to clean air. The repeat PAT testing was performed in a study participant after he/she was subjected to two venipuncture procedures (i.e. prior and after exposure), prolonged inactivity and fasting, dehydration and stress of being in the exposure chamber for a prolonged time period. A few studies have examined the effect of mental stress on PWA among adults with clinical coronary artery disease and have reported a greater than 20% reduction in PWA during stress among stress responders indicating on average, a peripheral vasoconstriction response to stress.[25-27] Thus, in the context of an exposure study design, the observed decrease in the PWA may be a result of effects of such unmeasured stressors associated with study designs and further exploration of this finding is required to ascertain if mental stress has any effect on PWA and consequently, on the PAT ratio. These findings also imply that PWA is sensitive to external stimuli and

changes in PWA as a result of an intervention should be cautiously interpreted in studies utilizing PWA as an outcome variable rather than a PAT ratio.

We obtained two different measures of reproducibility, namely CV and ICC. While ICC is a general measurement of agreement between repeated measurements on the same set of subjects, it represents the proportion of the total variance that is attributable to between-subject variability. It is a unitless quantity with a range of 0 to 1, where 0 indicates no reliability and 1 indicates perfect reliability. On the other hand, CV is a normalized measure of dispersion of a probability distribution, commonly used in biochemistry studies and it only provides a general idea regarding the spread around the mean. Since a CV does not take into account between-subject variability, an ICC is considered the most acceptable measure of agreement. The CV's observed in our study were in the range previously reported for the PAT ratio [3, 14, 15, 16], but the estimates showed a larger variation around the mean CV compared to the previous reports. Even though the estimates were fairly low and consistent with the prior literature, many researchers use an arbitrary value of ≤10% to indicate good reliability between the measurements [28], and by that definition, the CV's reported in our study were higher. However, it is reassuring that the CV's were also in the same range as those found in biochemical analyses of important variables used for clinical diagnosis and monitoring such as blood concentrations of cholesterol, glucose, and blood pressure. For example, CV's range from 5.3% to 17.3% for blood pressure [29-32], <5% to as high as 20% for blood glucose [33, 34], 3 to 5% for serum nitrate measurement [35] and 3% for total cholesterol and high HDL cholesterol [36] when measurements were obtained on the same or separate days. Blood flow in the fingertip vascular bed may be altered by the sympathetic and

autonomic nervous systems. Therefore, it is possible that changes in the finger PWA may occur because of changes in room temperature, mental stress, or other alterations in vascular tone. PAT testing is also highly sensitive to environmental factors such as temperature and noise. The CV's reported in our study presumably reflect variations in the peripheral arterial tone due to these extraneous factors. It may also be noted that the CV's for the PAT ratio from the pilot and CA exposure studies were not statistically different ($p=0.47$), suggesting that the setting of a controlled exposure study involving a complex battery of procedures compared to the pilot study did not further add to the variability of the repeated measurements of the PAT ratios.

In general, the ICC's for the PAT ratio obtained in our study were not in agreement with the fairly low CV's as the estimates were either low or negative. The magnitude of the ICC is dependent on the between-subject variability in the data.[37, 38] Even when all other things are equal, low levels of between-subject variability will reduce the magnitude of ICC even when the within-subject differences are small.[37, 38] We speculate that two factors contributed to low between-subject variability and consequently reduced the ICC in our study : (1) the PAT ratio estimates ranged from 1.0 to 3.5, with approximately 75% of the values below 2 and exhibited very little variation among young and otherwise healthy subjects, (2) in both settings, the subjects tended to be a homogenous population consisting of young, non-smoking and healthy college students with similar lifestyles and exposures. Additionally, the negative ICC estimate observed in the pilot study may suggest that the within-subject variability (or error) in the PAT ratio was much greater than the between-subject variability, perhaps due to different resting locations and activities chosen by the pilot study participants between the two

PAT tests. Although negative ICC estimates are rare, they are theoretically possible and have been previously reported for PAT ratios obtained on separate days.[15] There are three implications of low or negative ICC estimates: (1) the interpretation of repeated PAT measurements should be done with caution in young individuals, as these estimates indicate that two PAT measurements from a single subject vary almost as much as any randomly chosen measurements from two different subjects, (2) data on PAT measurements from a large number of subjects are required to detect changes in endothelial function if PAT ratio is the primary endpoint, and (3) the interpretation of a low ICC obtained in young CVD free individuals may not be applied to other populations such as older individuals with a known coronary artery disease or individuals with compromised endothelial function.

BAFMD is a more commonly used method for measurement of endothelial function and it is important to compare the reproducibility of the PAT technique with that of BAFMD. There is evidence supporting good reproducibility of the BAFMD technique within and between days.[39-41] While Welsch et al reported an ICC of 0.92 for FMD tests carried out on different days [39], Harris et al showed that repetitive reactive hyperemia over a 2-hour period had no effect on FMD measurements, with ICC's ranging from 0.45 to 0.81 for tests repeated within ½ hour, 1 and 2 hours on the same day.[40] Meirelles et al reported a CV and ICC of 5.8% and 0.70 for tests repeated on the same day respectively and 12.4% and 0.84 for tests repeated on separate days.[41] With an exception of these three studies, there also exists a great deal of discrepancy in the measures of reproducibility of BAFMD technique reported by several other studies, with CV's ranging from 6.7% to as high as 84% for measurements repeated on separate

days.[42-47] While the intra and inter day CV's for the PAT ratio observed in our study were lower than the CV's reported by most of the studies for the BAFMD technique, the observed ICC estimates were much lower than the previously reported range.[39-41] Thus, it may be concluded that the PAT device may not be a better alternative to the BAFMD technique when tests need to be repeated within a short interval of time.

It is important to note some of the strengths and limitations of our study before interpreting the results. To our knowledge, this is the first study that examined the reproducibility of the PAT technique in a controlled exposure study design in which participants were not only exposed to clean air, but also to other experimental procedures. We also reported the reproducibility of baseline PWA, in addition to the PAT ratio. Many variables known to acutely influence endothelial function were controlled for in our study designs. These variables included standardized time of day and room temperature used during PAT testing, limiting exercise, abstaining from alcoholic and caffeinated beverages 12 hours prior to the testing and overnight fasting. The testing procedure also calls for the standardization of posture, probe placement and a timed resting period to eliminate sympathetic stimulation prior to testing. Our study had several limitations. First, a small sample size produced less precise estimates for measures of reproducibility, especially in the pilot study. Second, the pilot study participants varied in their location of resting between the two PAT tests, a study design feature that could have been avoided. Third, we could not assess variations in the PAT measurements due to differences in temperature, gender, and menstrual phases in women. Fourth, the PAT measurements obtained from the exposure study participants were collected after a venipuncture procedure.

Further studies are warranted to examine the responsiveness of the PAT ratio to different environmental features and behavioral effects. Although we observed a low reproducibility of repeated PAT measurements, the device still may find its applicability in research studies involving repeated measurements if ICC's for the change in PAT ratios from pre to post obtained at two different sessions indicate good reproducibility. The study findings also imply that investigators may want to compare only post-intervention PAT ratios instead of the change from pre to post in the PAT ratio, or design studies where PAT testing is conducted prior to any other stress-inducing procedure.

In summary, we observed a lack of coherence between different statistical methods employed to examine the reproducibility of PAT measurements. However, based on the low ICC estimates obtained in the pilot and controlled exposure settings, we conclude that the PAT device does not produce reliable PAT ratio estimates when tests are repeated 2.5 hours apart on the same day and one week apart in young and healthy population. Therefore, prior to designing studies to examine the effects of interventions on endothelial function using this technique in young and healthy population, investigators: (1) should conduct their own carefully designed reproducibility studies in order to avoid the misinterpretation of repeated PAT measurements, (2) should be cautious while interpreting the effects of interventions on endothelial function based on a PAT ratio alone, (3)should consider examining a change in only post-intervention PAT ratio instead of examining a change from baseline to post-intervention in the PAT ratio. Further studies are required to examine if the change in the PAT ratios within a short time interval is reproducible on different days.

Tables

Table 1. Baseline Characteristics of Study Participants

Characteristic	Pilot study (Aim 1a)	Exposure study		
		Intra-CA day (Aim1b)	Inter-day (Aim 2)	
			Group 1	Group 2
N	10	48	36	50
Age (in Years)	28.7 (8.2)†	21.3 (3.1)	21.6 (3.4)	21.3 (3.0)
Gender (M:F)	9:1	38:10	30:6	40:10
Race*				
White	9 (90%)	38 (80.9%)	26 (74.3)	40 (81.6%)
Black	0 (0%)	1 (2.1%)	1 (2.8%)	1 (2%)
Asian	1 (10%)	8 (17%)	8 (22.9%)	8 (16.3%)
Ethnicity*				
Hispanic	0 (0%)	7 (14.9%)	6 (17.1%)	7 (14.3%)
Non-Hispanic	10 (100%)	40 (85.1%)	29 (82.9%)	42 (85.7%)
Systolic BP (in mm Hg)*	134.3 (13.7)	115.9 (12.1)	114.2 (11.5)	115.7 (12)
Diastolic BP (in mm Hg)*	73.0 (7.7)	70.2 (9.2)	70.2 (9.4)	69.9 (9.1)
Weight (in pounds)*‡		164.9 (36.3)	164.5 (34.5)	165.1 (35.5)
Body mass index (Kg/m^2)* ‡		25 (4.7)	24.6 (4.0)	25 (4.6)
Serum Cholesterol (mg/dL) ‡		156.0 (32.3)	157.8 (35.0)	155.2 (32.0)
HDL Cholesterol (mg/dL) ‡		51.8 (13.1)	49.5 (11.1)	51.3 (13.0)
LDL Cholesterol (mg/dL) ‡		82.4 (29.6)	86.6 (31.1)	82.2 (29.0)
Serum triglyceride (mg/dL) ‡		108.9 (65.5)	108.6 (63.5)	108.4 (64)

* Missing data on the exposure study subjects on the variables marked with *.
† Values represent mean (SD) unless otherwise specified.
‡ Information on these variables was not collected for pilot study subjects.

Table 2. Pre to Post Changes in the Mean PAT Measurements in the Pilot and Clean Air Exposure Studies

Study/Time Point	N	PAT Ratio Mean ± SD	$PWA_{Control}$ Mean ± SD	$PWA_{Occlusion}$ Mean ± SD
Pilot Study				
First PAT test	10	1.65 ± 0.3	945.6 ± 405.0	941.0 ± 343.9
Repeat PAT test	10	1.82 ± 0.6	752.7 ± 406.9	818.6 ± 404.1
Diff (Repeat-First)	10	0.18 ± 0.7	-192.8 ± 271.6	-122.4 ± 340.4
p-value†		0.45	0.051	0.28
CA Exposure Study				
Pre CA PAT test	48	1.65 ± 0.4	605.5 ± 428.8	616.5 ± 404.8
Post CA PAT test	48	1.86 ± 0.5	187.5 ± 160.3	192.8 ± 157.8
Diff (Post-Pre)	48	0.21 ± 0.5	-418.0 ± 376.4	-423.7 ± 374.8
p-value†		0.005	<0.0001	<0.0001

† For comparisons of PAT indices within the same day using a paired t-test
PWA, Pulse wave amplitude; CA, Clean air

Table 3. Comparison of PAT Measurements from the Pilot and CA Exposure Studies

Time Point	Study	N	PAT Ratio Mean ± SD	$PWA_{Control}$ Mean ± SD	$PWA_{Occlusion}$ Mean ± SD
First PAT test	Pilot	10	1.65 ± 0.3	945.6 ± 405.0	941.0 ± 343.9
Pre CA PAT test	CA exposure	48	1.65 ± 0.4	605.5 ± 428.8	616.5 ± 404.8
p-value†			0.99	0.03	0.02
Repeat PAT test	Pilot	10	1.82 ± 0.6	752.7 ± 406.9	818.6 ± 404.1
Post CA PAT test	CA exposure	48	1.86 ± 0.5	187.5 ± 160.3	192.8 ± 157.8
p-value†			0.86	<0.0001	<0.0001

† For comparisons of PAT indices from two different experiments using an independent sample t-test
CA, Clean air; PWA, Pulse wave amplitude

Table 4. Measures of Intra-day Reproducibility of PAT Measurements

Measure of Reproducibility	N	PAT Ratio Estimate (95%CI)	$PWA_{Control}$ Estimate (95%CI)	$PWA_{Occlusion}$ Estimate (95%CI)
Intra-subject CV† (%)				
Pilot study	10	19.8 (8.3, 31.2)	30.9 (6.7, 55.2)	30.7 (5.1, 56.2)
CA Exposure Study	48	16.7 (13.5, 19.9)	72.7 (63, 82.4)	70.4 (60, 80.8)
ICC‡				
Pilot study	10	-0.07 (-0.63, 0.55)	0.70 (0.20, 0.92)	0.58 (-0.004, 0.87)
CA Exposure Study	48	0.40 (0.14, 0.61)	-0.06 (-0.34, 0.22)	-0.14 (-0.41, 0.14)

CI, Confidence Interval; CA, Clean air; PWA, Pulse wave amplitude

† CV, Coefficient of variation expressed as %

‡ ICC, Intra-class correlation coefficient

Table 5. Pre to Post Changes in the Mean PAT Measurements by the Order of Visit in the Clean Air Exposure Study

Time Point	N	PAT Ratio Mean ± SD	$PWA_{Control}$ Mean ± SD	$PWA_{Occlusion}$ Mean ± SD
CA as Visit 1‡				
Pre CA PAT test	19	1.61 ± 0.3	431.8 ± 399.6	458.0 ± 389.9
Post CA PAT test	19	1.68 ± 0.4	156.0 ± 140.9	164.7 ± 150.8
Diff (Post-Pre)	19	0.07 ± 0.4	-275.8 ± 363.4	-293.3 ± 361.5
p-value†		0.43	0.004	0.002
CA as Visit 2‡				
Pre CA PAT test	17	1.66 ± 0.5	718.6 ± 362.9	687.8 ± 351.9
Post CA PAT test	17	1.82 ± 0.5	183.8 ± 168.6	209.7 ± 196.3
Diff (Post-Pre)	17	0.16 ± 0.6	-534.2 ± 345.5	-478.1 ± 338.4
p-value†		0.26	<0.0001	<0.0001
CA as Visit 3‡				
Pre CA PAT test	12	1.67 ± 0.5	721.2 ± 496.5	766.5 ± 442.0
Post CA PAT test	12	2.18 ± 0.6	242.6 ± 175.9	213.5 ± 104.0
Diff (Post-Pre)	12	0.50 ± 0.5	-478.5 ± 394.8	-553.0 ± 409.3
p-value†		0.002	0.002	0.0007

CA, Clean Air; PWA, Pulse wave amplitude

† For comparisons of PAT indices within the same day using a paired t-test

‡ Data from the CA exposure study were categorized by the order of visit (Visit 1, Visit 2, and Visit 3)

Table 6. Measures of Intra-day Reproducibility of PAT Measurements by the Order of Visit in the Clean Air Exposure Study

Measure of Reproducibility	N	PAT Ratio Estimate (95%CI)	$PWA_{Control}$ Estimate (95%CI)	$PWA_{Occlusion}$ Estimate (95%CI)
Intra-subject CV† (%)				
Visit 1§	19	13.0 (8.9, 17.1)	68.5 (53.2, 83.7)	67.4 (51.2, 83.6)
Visit 2§	17	18.6 (13.2, 24.1)	81.7 (62.2, 101.2)	75.2 (54.8, 95.6)
Visit 3§	12	19.9 (11.1, 28.8)	66.7 (48.3, 85.1)	68.3 (45.7, 91.0)
ICC‡				
Visit 1§	19	0.53 (0.13, 0.79)	0.06 (-0.39, 0.49)	0.02 (-0.42, 0.46)
Visit 2§	17	0.29 (-0.20, 0.66)	-0.33 (-0.69, 0.16)	-0.23 (-0.62, 0.26)
Visit 3§	12	0.39 (-0.19, 0.77)	-0.04 (-0.51, 0.57)	-0.30 (-0.72, 0.29)

CI, Confidence Interval; PWA, Pulse wave amplitude

† CV, Coefficient of variation expressed as %

‡ ICC, Intra-class correlation coefficient

§ Data from the CA exposure study were categorized by the order of visit (Visit 1, Visit 2, and Visit 3)

Table 7. Changes in the Mean PAT Measurements by Visit

Time Point	N	PAT Ratio Mean ± SD	$PWA_{Control}$ Mean ± SD	$PWA_{Occlusion}$ Mean ± SD
Group 1				
First visit	36	1.64 ± 0.4	544.0 ± 408.6	526.4 ± 368.3
Second visit	36	1.66 ± 0.5	611.4 ± 454.5	593.9 ± 413.5
Third visit	36	1.75 ± 0.5	643.5 ± 425.4	663.5 ± 407.1
p-value†		0.53	0.32	0.16
Group 2				
First visit	50	1.61 ± 0.4	512.8 ± 416.6	514.1 ± 384.5
Second visit	50	1.60 ± 0.5	592.4 ± 451.0	576.3 ± 415.2
Diff (Second-First)	50	-0.01 ± 0.5	79.6 ± 330.1	62.2 ± 333.3
p-value‡		0.86	0.09	0.19

† For comparisons of PAT indices using a repeated measures ANOVA

‡ For comparisons of PAT indices using a paired t-test

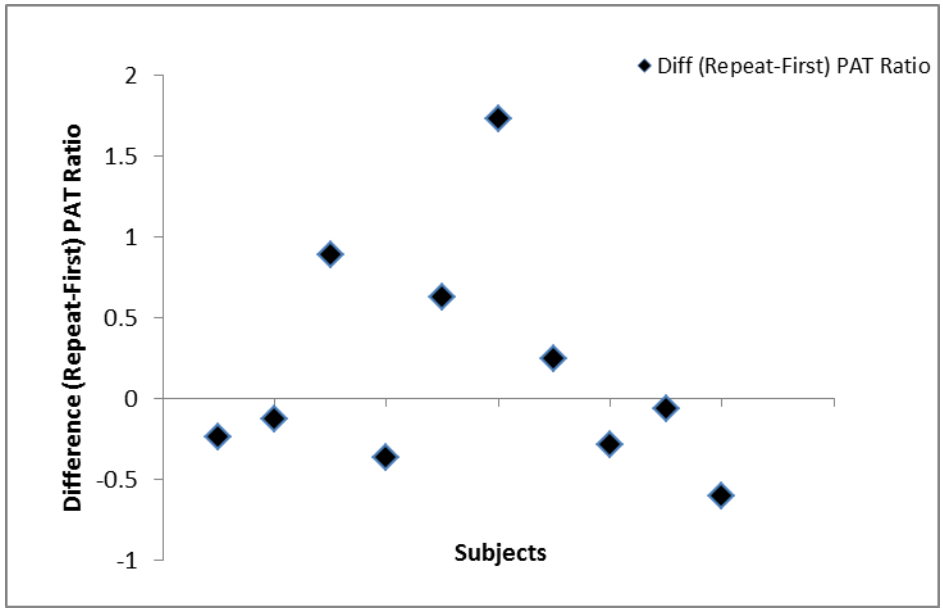
Table 8. Measures of Inter-day Reproducibility of PAT Measurements

Measure of Reproducibility	N	Number of visits*	PAT Ratio	$PWA_{Control}$	$PWA_{Occlusion}$
Intra-subject CV (%)	50	2 or 3	17.1 (13.7, 20.6)	40.3 (31.2, 49.4)	40.4 (32.2, 48.6)
ICC for group 1	36	3	0.27 (0.07, 0.49)	0.70 (0.54, 0.82)	0.60 (0.42, 0.75)
ICC for group 2	50	2	0.43 (0.18, 0.63)	0.70 (0.53, 0.82)	0.65 (0.46, 0.78)

*The number of visits completed by each subject varies from 2 to 3.
CV, Coefficient of variation expressed as %; ICC, Intra-class correlation coefficient
Values represent estimates and their 95% CI's.

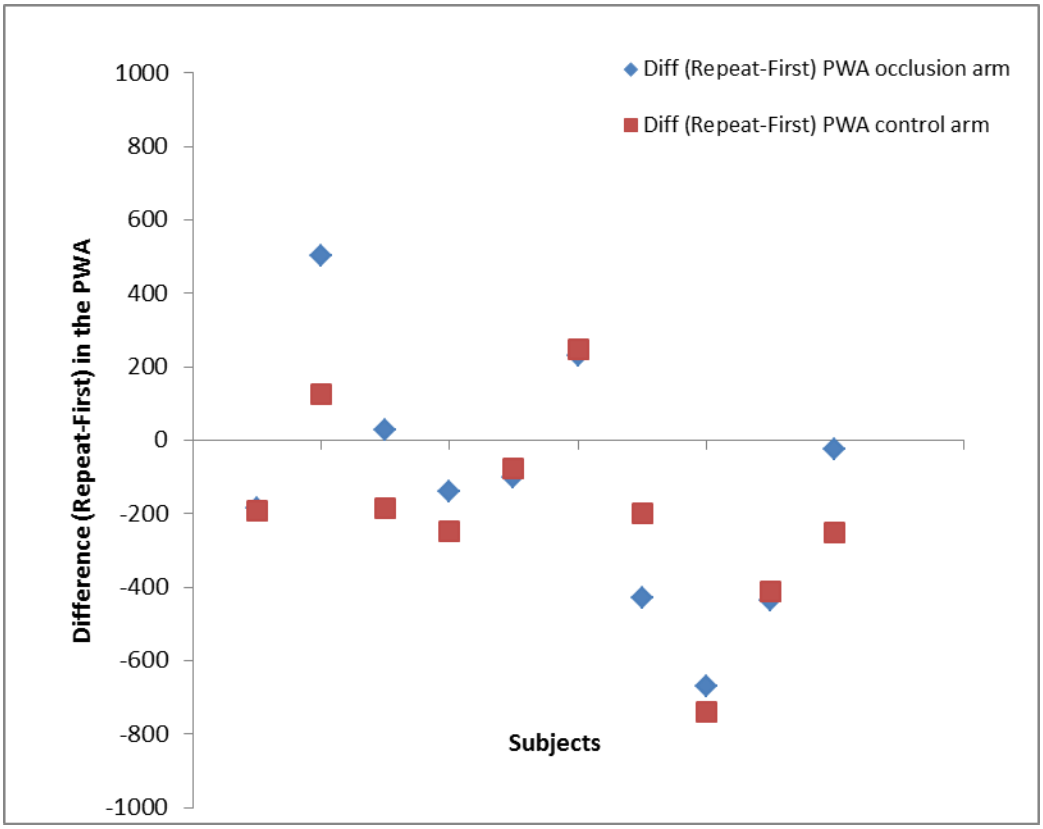
Figures

Figure 1. Differences in the PAT ratio from Repeat to First Test in the pilot study



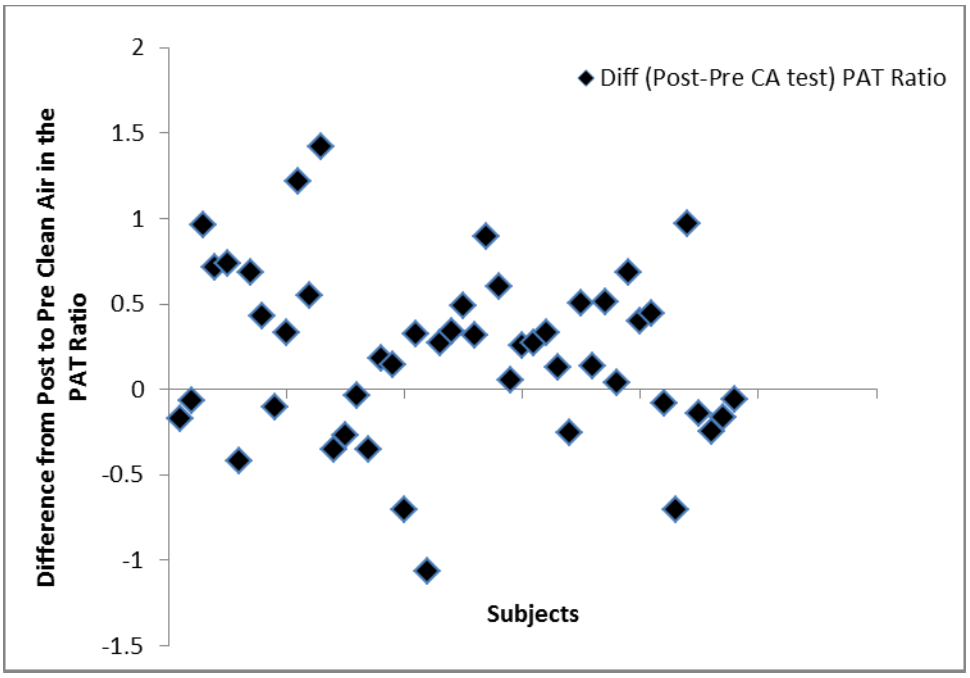
First PAT ratio=PAT ratio obtained from the first PAT test
Repeat PAT ratio= PAT ratio obtained from the repeat PAT test
Diff (repeat-first) PAT ratio=Difference between the repeat and the first PAT ratio

Figure 2. Differences in the PWA for both arms from Repeat to First PAT test in the pilot study



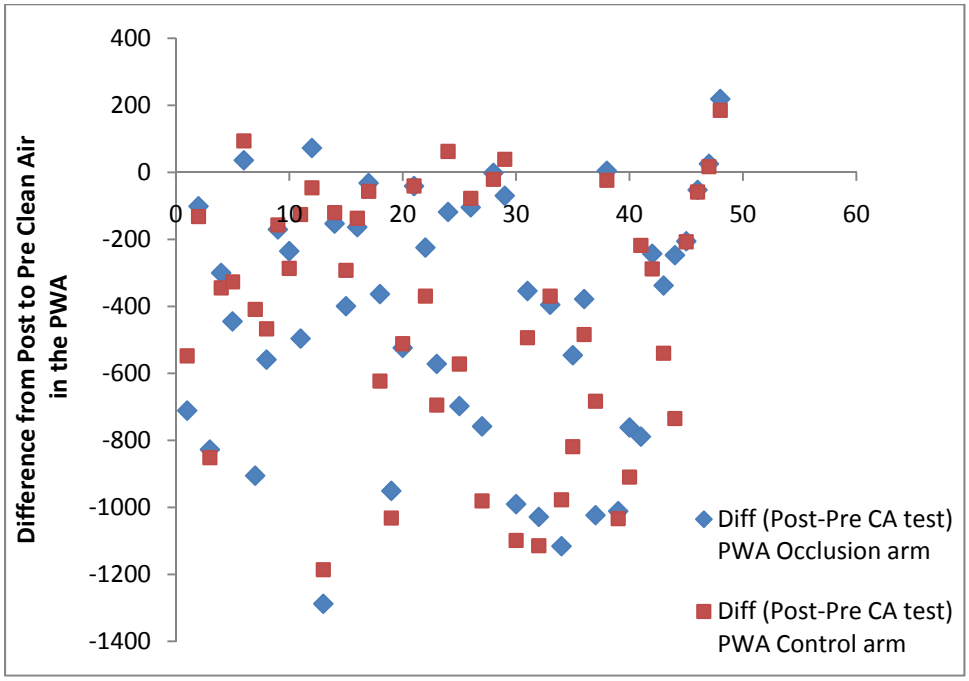
First PWA=Baseline PWA obtained from the first PAT test
Repeat PWA= Baseline PWA obtained from the repeat PAT test
Diff (repeat-first) PWA=Difference between the repeat and the first PWA

Figure 3. Differences in the PAT ratio from Post to Pre Clean Air PAT Test in the CA exposure study



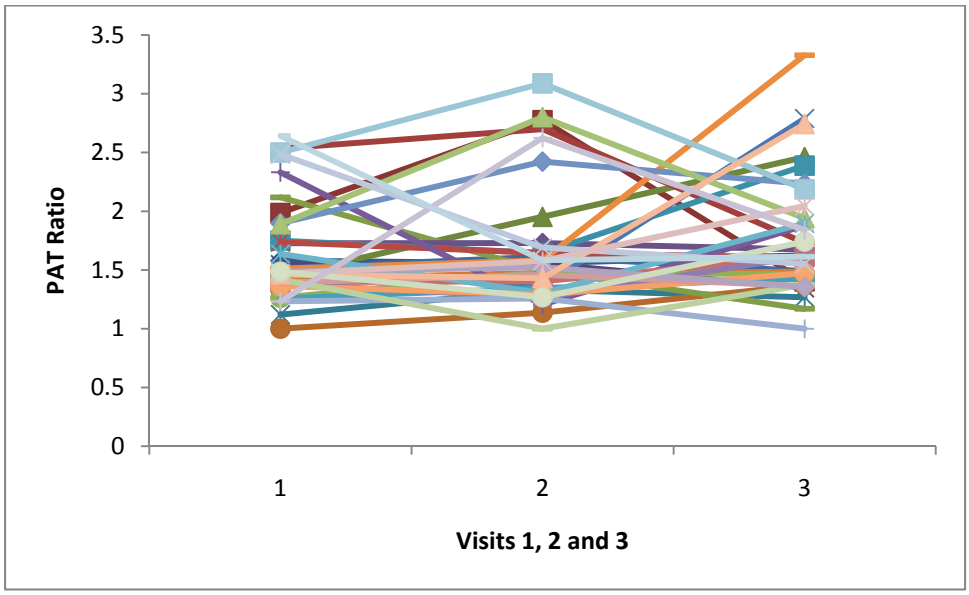
Pre CA PAT ratio=PAT ratio obtained before the exposure to clean air (CA)
Post CA PAT ratio= PAT ratio obtained after the exposure to CA
Diff (post-pre) PAT ratio=Difference between the PAT ratios obtained before and after the exposure to CA

Figure 4. Differences in the PWA for both arms from Post to Pre Clean Air PAT Test in the CA exposure study



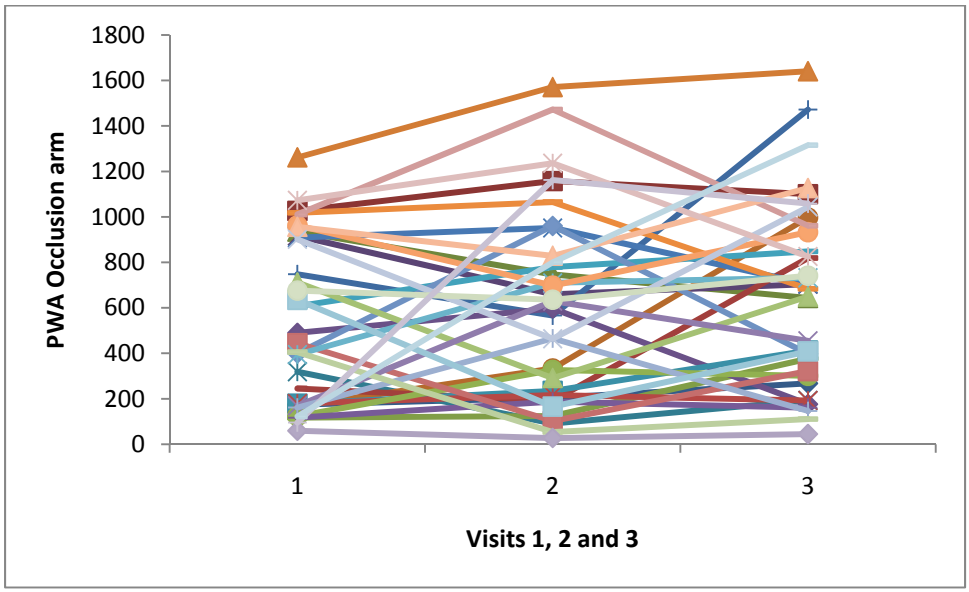
Pre CA PWA=Baseline PWA obtained before the exposure to clean air (CA)
Post CA PWA= Baseline PWA obtained after the exposure to CA
Diff (post-pre) PWA=Difference between the basleine PWA's obtained before and after the exposure to CA

Figure 5. Variation in the PAT Ratios by Visit in the 36 Subjects from Group 1



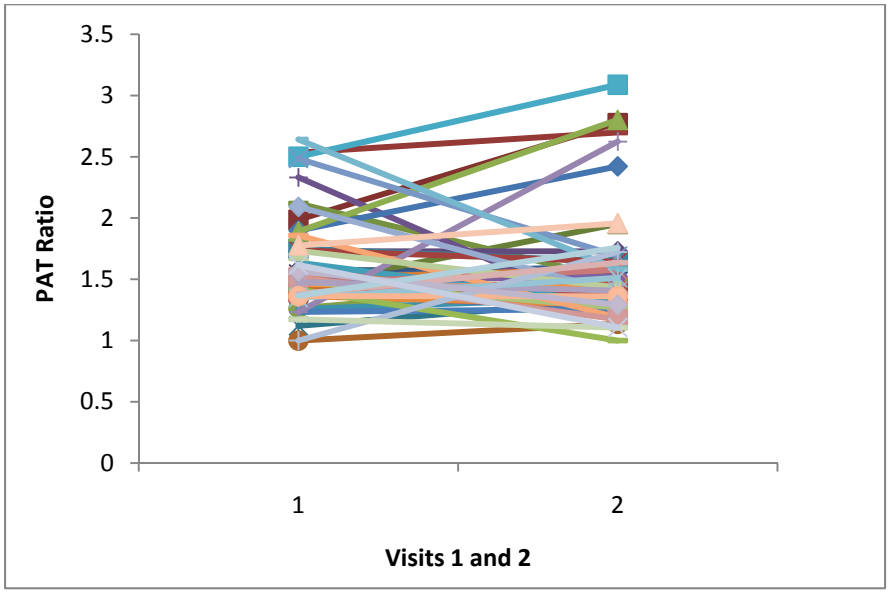
Each line represents an individual subject.

Figure 6. Variation in the Pulse Wave Amplitude of the Occlusion Arm by Visit in the 36 Subjects from Group 1



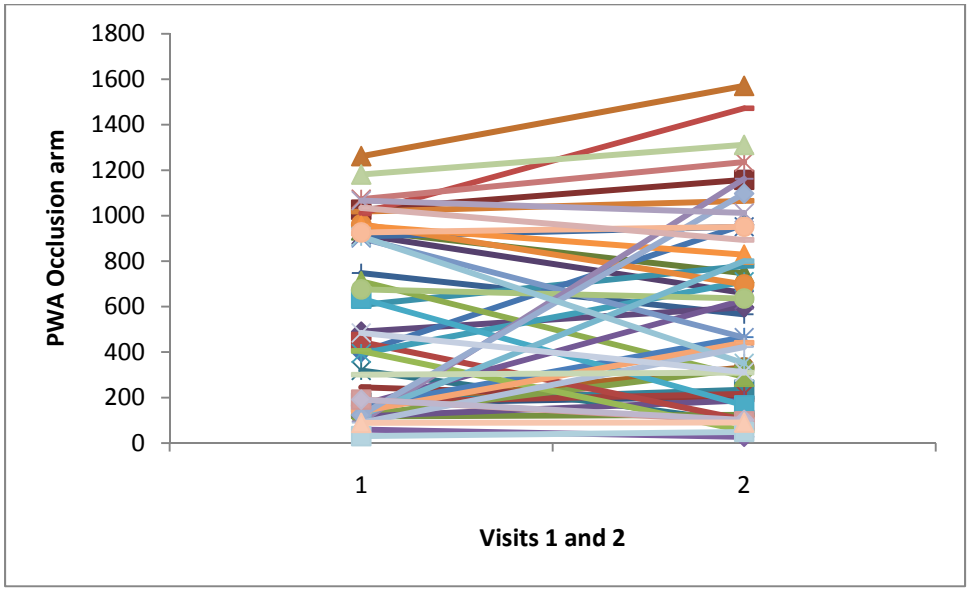
Each line represents an individual subject.

Figure 7. Variation in the PAT Ratios by Visit in the 50 Subjects from Group 2



Each line represents an individual subject.

Figure 8. Variation in the Pulse Wave Amplitude of the Occlusion Arm by Visit in the 50 Subjects from Group 2



Each line represents an individual subject.

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VALIDATION OF ENDOPAT TECHNOLOGY

PART TWO: EFFECTS OF MENTAL STRESS ON ENDOPAT MEASUREMENTS

by

SAMPADA GANDHI

Manuscript 1 (Part 2) of 3 of a dissertation entitled

EFFECTS OF AIR POLLUTANTS ON ENDOTHELIAL FUNCTION

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University of Medicine and Dentistry of New Jersey

School of Public Health

Written under the direction of

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ABSTRACT OF MANUSCRIPT 1 (PART 2) OF 3

EFFECTS OF MENTAL STRESS ON ENDOPAT MEASUREMENTS

Dissertation Director:

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ABSTRACT

Objectives: We sought to examine the changes in the baseline pulse wave amplitude (PWA) during and after the completion of a mental stress task (MST) from its pre-stress baseline and the effects of the MST on PAT ratio in young and healthy individuals.

Methods: Nine healthy participants between the ages of 18-45 years underwent a study protocol consisting of a baseline (or pre-MST) PAT test using a reactive hyperemia procedure, a 5-minute mental arithmetic stress task, a 10-minute rest, and a post-MST PAT test. The changes in the PWA during and 10-minutes after the completion of the MST from its baseline were examined using repeated measures analysis of variance, and the changes in the PAT ratio from pre to post MST were examined using a paired t-test.

Results: The PWA decreased significantly during the MST compared to its pre-MST baseline in both arms. An initial stress response as indicated by decrease was observed during the first 30 seconds of the MST in 8 subjects (PWA of occlusion arm: -257.2; 95% CI:-335.9, -178.4) and thereafter the subjects adapted to stress by either remaining lower or by gradually returning to their pre-MST baseline. Although the 10-min post-MST PWA was non-significantly lower compared to its pre-MST baseline, it suggested a trend

toward a sustained decrease. The MST did not produce substantial changes in the PAT ratio.

Conclusions: Mental stress decreases PWA, with the greatest decrease observed within the first 30 seconds. Larger studies are required to examine if the decrease is sustained, and if the response to stress varies with tasks different in duration and intensity of stress.

EFFECTS OF MENTAL STRESS ON ENDOPAT MEASUREMENTS

Introduction

In the first part of the manuscript one, we observed two novel findings with respect to pulse wave amplitude (PWA) in the pilot study and the clean air (CA) exposure study: (1) the baseline PWA of the pre-CA PAT test in the CA exposure study was significantly lower than the baseline PWA of the first PAT test of the pilot study and of the three exposure visits, it was the lowest on subjects' first visit as opposed to the second or third visit. Unlike the pilot study design, the participants from the exposure study were subjected to venipuncture, a known painful stressor, approximately 10 minutes prior to the PAT testing and were more anxious in anticipation of being exposed to air pollutants in the exposure chamber, especially on their first visit, (2) the baseline PWA of the post-CA PAT test was also significantly lower compared to the baseline PWA of the pre-CA PAT test. Since venipuncture always preceded PAT testing, the participants were subjected to two venipuncture procedures before the baseline PWA of the post-CA PAT test was obtained. Both these findings suggest a possible role of mental stress in causing a sustained decrease in the PWA that can be detected by a PAT device even after the elimination of the stressful procedure responsible for the decrease.

A few studies have examined the effects of a mental stress task (MST) on endothelial function using the PAT device in patients with coronary artery disease (CAD).[1-4] These studies were undertaken to examine the changes in the PWA during the task performance and thereby, to assess if the device can detect mental stress-induced myocardial ischemia. These studies reported a characteristic PAT signal response, with

diminution of the PWA obtained during stress from its baseline. However, no studies have examined if the decrease in the PWA observed during stress remains persistent even after the task is completed. There is also limited evidence regarding the effects of stress on the PAT ratio obtained following the completion of a MST.[5-6] To address these questions, we sought to examine (1) the changes in the PWA during and 10-minutes after the completion of a MST from its baseline; (2) the effects of a MST on PAT ratio in young and healthy individuals.

Materials and Methods

Study Subjects

Nine healthy non-smoking subjects between the ages of 18-45 years were recruited from the Rutgers University community using advertisement flyers. When a potential candidate contacted a research staff, he/she was screened to ascertain his/her eligibility to participate in the study using a standard telephone questionnaire. Subjects were excluded if they indicated presence of cardiovascular disease, cancer, stroke, hypertension, asthma, neurological disease, and kidney or liver disease. Additional exclusions were: pregnant or breastfeeding females, subjects who were taking daily anti-hypertensive medications or any other vaso-active medications, and subjects with a known allergy to latex. The study was conducted at the Clinical Center of the Environmental and Occupational Health Sciences Institute (EOHSI) in Piscataway, New Jersey and it was approved by the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School Institutional Review Board.

Study Protocol

Candidates deemed eligible to take part in the study were scheduled for one session to be conducted between 8:00 - 10:00 A.M. Subjects were asked to arrive following an overnight fast beginning at 12:00 midnight before the day of testing. They were asked to avoid consumption of alcoholic and/or caffeinated beverages and vasoactive medications on the day of testing. Upon subject's arrival at the EOHSI Clinical center at 8 A.M., a written informed consent was obtained. The research staff performed a check-in to ascertain if subjects have followed the above-mentioned instructions. A pregnancy test was administered to women**.** Each subject underwent an approximately 50-minute experimental protocol consisting of a baseline (or pre-MST) PAT test using a reactive hyperemia procedure, a 5-minute mental arithmetic stress task, a 10-minute resting interval and a post-MST PAT test (Figure 1). Subjects remained fasting until the post-MST PAT test was completed.

Reactive Hyperemia or PAT Testing Protocol

The PAT testing was conducted in a dark, noise and temperature controlled room (72 deg. F). An experienced technician who received training from the Itamar Medical representative conducted these tests. Subjects were asked to turn off cellular phones and to avoid talking. Subjects relaxed in a supine position with both their arms resting on arm support pads. Latex covered finger probes were applied on the tip of each index finger for measurement of PWA. Three readings of the subject's blood pressure were recorded using a digital blood pressure monitor (Omron automatic blood pressure monitor, Model HEM-705CP) and entered into the PAT device. A blood pressure cuff was then applied

on the subject's non-dominant arm (or occlusion arm) for the purpose of a 5-minute occlusion of the blood flow. The subject's dominant arm was referred to as a control arm. After a 5-min rest period, beat-by-beat PWA signals were collected at baseline for approximately 5 minutes following which the blood pressure cuff was inflated to at least 50 mm Hg above the subject's systolic blood pressure for 5 minutes. PWA signals were collected continuously during the 5-minute occlusion and for a period of at least 5 minutes following cuff deflation. A description of the methods used for data collection has been provided in the Appendix of this dissertation.

Mental Stress Task (MST) Protocol

Following the baseline PAT test, a research staff member other than the one who performed the check-in procedures and PAT testing administered instructions to the subjects to perform a mental arithmetic stress task. The task was then commenced by asking the subject to perform serial subtractions under moderate harassment starting at the number 2,083. The subjects were asked to count aloud backwards from 2083 to zero by subtracting in number 13 for a period of 5 minutes. They were asked to calculate as quickly and accurately as possible and were instructed that they would be judged on time taken to calculate and accuracy of calculations. In instances where a subject miscalculated, he/she was asked to start all over again. This mental arithmetic stress task has been widely used in previous research. [1, 6-10]

Study Variables

The PWA was measured thirteen times in each subject as follows: [1] at baseline prior to the MST or baseline of the pre-MST PAT test (time 1) [2] just prior to the beginning of the MST (time 2) [3] every 30 seconds during the 5-min MST (time 3-time 12), and [4] 10-minutes after the completion of the MST (time 13). A pre and post-MST PAT ratios were obtained. A description of the study variables is provided in Table 1 and Figure 1.

Statistical Analyses

Baseline characteristics of the study subjects such as age, gender, and race were reported. Mean and standard deviation of the PWA measurements obtained at time 1 to time 13 were reported for the occlusion and the control arm separately. Comparisons of serial PWA measurements were made using a linear mixed model for repeated measures analysis of variance (ANOVA) to test if the PWA measurements obtained during (time 3-12) and after the completion of the MST (time13) were different from the PWA obtained at time 1. A correlation structure with the lowest Akaike's Information Criterion (AIC) was selected for the linear mixed model. A paired t-test was performed to determine if the two PAT ratios (i.e. pre and post-MST PAT ratios) were different.

Next, to examine if the change in the PWA from time 1 to time 13 was significantly different among the subjects who exhibited an initial decrease of ≥ 20% from their PWA at time 1 compared to the subjects who exhibited an initial decrease of <20%, a "stress ratio" was calculated for both arms by dividing the PWA obtained within the first 30 seconds of the MST (time 3) by the PWA obtained at time 2 (Baseline PWA_{MS}). The time interval of the first 30 seconds of the MST was selected as it represented a subject's initial response to stress. Based on the stress ratio, subjects were

classified into two groups: (1) a subject with a stress ratio less than or equal to 0.8 (≥ 20% decrease) was considered a 'Stress Responder', (2) a subject with a stress ratio greater than 0.8 (<20% decrease) was considered a 'Stress Non-responder'. The cut-off of 0.8 was used based on prior practice.[1, 6]

A paired t-test was conducted to determine if the PWA obtained at time 13 was different from the PWA obtained at time 1 among stress responders and non-responders separately. Two-sample t-tests were conducted to determine if the PWA obtained at time 1, time 2, time 3, and time 13 were different among stress responders and non-responders. Two-sample t-tests were also conducted to determine if the mean difference between the PWA obtained at time 13 from time 1 were different between the responders and non-responders. Similarly, a paired t-test was performed to determine if the two PAT ratios (i.e. pre and post-MST PAT ratios) were different among stress responders and non-responders. Two-sample t-tests were conducted to determine if the PAT ratio obtained prior to the MST, post-MST and the difference from post to pre-MST were different among stress responders and non-responders.

A sensitivity analysis was performed by replacing the PWA at time 3 by the mean PWA during the MST. Another stress ratio was calculated by dividing the mean PWA during the MST by the PWA obtained at time 2 (Baseline PWA_{MS}). Based on this new stress ratio, subjects were classified into responders and non-responders using the same cut-off of 0.8. All PWA comparisons were repeated for the responders and non-responders. All analyses were conducted using the SAS programming language version 9.3 (SAS Institute, Inc, Cary, NC).

Results

Baseline Characteristics of Study Subjects

The study subjects were predominantly white with a mean age of 28.3 ± 8.6 years. Five out of the 9 enrolled were males. Blood pressure and body mass index were within a normal range (Table 2).

Changes in the PWA

The mean (± Standard deviation) PWA measurements for the occlusion and the control arm obtained at baseline (time 1), prior to the beginning of the MST (time 2), during the MST (time 3-12), and 10-minutes after the completion of the MST (time 13) are presented in Table 3. The mean PWA decreased from time 1 to time 2 (Occlusion arm: -67.8; 95% CI: -123.6, -11.9). This decrease corresponded with the subject taking instructions from the research staff regarding the MST. The mean PWA showed the greatest decline from time 2 to the first 30 seconds of the MST (time 3) (Occlusion arm: --257.2; 95% CI: -335.9, -178.4). Thereafter, the mean PWA obtained at time 4 to time 12 continued to show a consistent decrease compared to the PWA at time 1. The mean PWA at time 13 was also lower compared to the PWA at time 1. Although the decrease was observed consistently in both the arms, the mean PWA's of the control arm were lower compared to the mean PWA's of the occlusion arm at all times (Table 3). The response to stress by the occlusion arm may be lower compared to the control arm due to the fact that the occlusion arm underwent a blood pressure cuff occlusion for 5 minutes as a part of the pre-MST PAT test. Figures 2 and 3 depict the changes in the PWA from time 1 to time 13 in individual subjects for the occlusion and the control arm respectively. Out of

the 9 enrolled, 8 subjects showed an initial decline from time 2 to 3 in both arms. Of those, 5 subjects remained lower throughout the 5 minute MST while 3 showed a gradual increase after the initial decrease. One subject showed an increase in the PWA throughout the 5-min MST (Figures 2 and 3).

Table 4 shows the comparison of the changes in the PWA obtained during and after the MST with its baseline using repeated measures ANOVA. The PWA obtained at all times i.e. from time 2 to time 12 was significantly lower from the PWA obtained at time 1, with an exception of the PWA obtained in the last 30 seconds of the MST for the occlusion arm. The effect estimate comparing the change in the PWA in the first 30 seconds during the MST (time 3) with time 1 was the greatest for both arms, indicating that the effect of the MST on PWA was the strongest during that time interval. The decrease in the PWA was greater in the control arm at all times compared to the occlusion arm. For both arms, the PWA obtained 10-mins after the completion of the MST remained lower compared to time 1, indicating that the PWA did not return to its baseline even after 10-minutes following the completion of the MST, but the effects were not significant ($p=0.07$ for the occlusion arm, $p=0.13$ for the control arm).

Table 5 shows the computation of the stress ratio for each subject. When the stress ratio was computed by dividing the PWA at time 3 by PWA at time 2, five subjects were classified as stress responders and 4 subjects were classified as stress non-responders. When the stress ratio was computed by dividing the mean PWA during MST by PWA at time 2 as a part of the sensitivity analysis, the results matched with the earlier method,

except for one subject, who was classified as a non-responder instead of a responder. Figure 4 shows a typical PWA tracing from a stress-responder and a non-responder. Figures 5 and 6 depict the changes in the PWA of the occlusion arm from time 1 to time 13 in the responders and non-responders using the first method (i.e. PWA at time 3).

Table 6 shows the comparison of the PWA's of the occlusion arm within a responder or a non-responder group as well as between the two groups. The mean PWA at time 1, time 2, time 3 and time 13 was lower in the responders compared to the non-responders, but the difference between the two groups was not significant (Table 6). The PWA at obtained at time 13 was lower compared to the PWA at time 1, but the decrease was not statistically significant in both responders and non-responders (Responders: p=0.19, Non-responders: p=0.33). The decrease in the PWA from time 1 to time 13 was greater among the non-responders but not significantly different from the decrease observed in the responders (Responders: -165.4 ± 236.7; Non-responders:-188.4 ± 328.4, p=0.91). Table 7 shows similar comparisons made using the PWA of the control arm. The results obtained from the control arm were in line with the occlusion arm.

When the response to stress was defined using the mean PWA during MST, the results were consistent with the results obtained from the first method i.e. using PWA at time 3. The mean PWA of the occlusion arm at time 1, time 2, time 3 and time 13 was lower in the responders compared to the non-responders, but the difference between the two groups was not significant ($p>0.05$). The PWA of the occlusion arm obtained at time 13 was lower compared to the PWA at time 1, but the decrease was not statistically

significant in both responders and non-responders (Responders: p=0.26, Non-responders: p=0.27). The decrease in the PWA of the occlusion arm from time 1 to time 13 was greater among the responders but not significantly different from the decrease observed in non-responders (Responders: -187.1 ± 267.5; Non-responders: -166.4 ± 288.6, p=0.92).

Changes in the PAT Ratio

Table 8 shows the changes in the mean PAT ratio. The mean PAT ratios showed a slight increase from pre to post-MST (1.63 ± 0.5 versus 1.79 ± 0.4). However, the two PAT ratios were not significantly different one another (p=0.39). Figure 7 shows the pre and post-MST PAT ratios for individual subjects. When stratified by the responder status, a slight increase was observed among both the responders and the non-responders. However, the two PAT ratios were not significantly different in neither of the two groups (p=0.72 for responders, p=0.17 for non-responders) (Table 8).

Discussion

In a pilot study conducted to examine the changes in pulse wave amplitude and PAT ratio during and after completion of a mental stress stask, we found that: (1) the PWA decreased significantly prior to the beginning of the MST (time 2) and during the MST (time 3-12) compared to the pre-MST PWA (time 1) in both arms ($p<0.0001$), (2) an acute stress response was observed during the first 30 seconds of the MST in both arms, showing the greatest decrease from time 1 ($p<0.0001$ for both arms), (3) the subjects adapted to mental stress differently. Eight subjects of the 9 enrolled showed an initial

decrease in the PWA in the first 30 seconds of the MST. Of those, 5 remained lower throughout the 5-min stress period compared to its pre-MST baseline while 3 subjects showed a gradual increase in the PWA after the initial decrease. One subject showed an increase throughout the stress period, (4) although the PWA obtained at 10-mins after the completion of the MST (time 13) was not significantly lower compared to the PWA at time 1, it suggested a trend towards a persistent decrease that can be detected by the PAT device, (5) although the PWA obtained at time 13 was lower than the PWA at time 1 in both stress responders and non-responders, the decrease was not significantly different between the two groups, and (6) mental stress did not affect the PAT ratio significantly. The mean PAT ratios showed a non-significant increase from pre-MST to post-MST. This increase was observed even when the subjects were classified into responders and non-responders.

Our study concluded that mental stress results in a decrease in PWA or increase in peripheral vascular tone. This vasoconstriction response as reflected by a decrease in PWA has been previously demonstrated by investigators in patients with CAD[1-4] and in healthy adults.[5, 6] Thus, our finding strongly agrees with prior research. Our study further showed that an acute response to mental stress occurred within the first 30 seconds during the stress task performance and thereafter, the subjects adapted to the effects of stress by either remaining lower throughout the 5-min stress period or by slowly returning to the pre-stress baseline. Another study by Hassan et al (2009) reported a similar "initial or early" response to stress where participants were given 2 minutes to prepare their speech and 3 minutes to speak as part of a public speaking stress task.[4] In this study, the reduction in PWA during the 2-minute speech preparation period was

significantly more pronounced than during the speaking task, suggesting that the effect of mental stress on PWA occurs as soon as preparation for a stress task commences and individuals adapt to the effects of stress thereafter. Using a brachial artery flow-mediated dilation (BAFMD) technique, Harris et al (2000) also reported a significantly lower baseline brachial artery diameter during mental stress compared to its pre-stress baseline where the effects were observed within the first 30 seconds of the stress task.[11] This instantaneous response to mental stress may be attributed to the activation of sympathetic nervous system (SNS) and release of catecholamines as described in the next paragraph.

Mental stress can provoke a significant sympathetic response [12-14] and thereby cause an increase in plasma catecholamines, namely, epinephrine and norepinephrine as reported in the previous literature. [2, 7, 8, 15-18] Due to the activation of the SNS, there is a rapid rise in blood pressure and heart rate.[1, 12, 13, 16, 17] Upon release in the blood stream, the catecholamines constrict blood vessels and the effects are observed rapidly within 4 to 8 seconds of their release.[19] There is also direct evidence that mental stress causes vasoconstriction in visceral arteries such as renal and superior mesenteric in humans and increases mean arterial pressure, heart rate and vascular resistance from the first minute of stress task.[20] The mechanism of causing an increase in systolic and diastolic blood pressure and heart rate via activation of sympathetic nervous system (SNS) during mental stress has been documented widely in animal and human studies.[1, 12, 13, 16, 17] Although we didn't collect data on blood pressure and heart rate changes during mental stress, these studies provide substantial evidence suggesting a link between mental stress and SNS activation.

In the part one of this manuscript, we observed a significant decrease in the baseline PWA from pre to post clean air exposure. The baseline PWA of the pre-CA PAT test in the exposure study participants was also significantly lower compared to the baseline PWA of the first PAT test in the pilot study participants. The exposure study participants underwent venipuncture which is a known painful stressor[15] approximately 10 minutes prior to the PAT testing and in addition, they presumably experienced psychological stress from anticipation of being exposed to air pollutants in the exposure chamber. In addition to being a stressor, venipuncture procedure may also be responsible for causing an increase in the vascular tone via release of clotting factors such as serotonin[21], thromboxane A2[22] and platelet activating factor. [23-24] Thus, based on the findings of this study, mental stress may have played a substantial role in causing the observed decrease in the PWA. Although, we failed to provide evidence that the effects of mental stress task remain persistent even after the task is completed, we observed a trend toward a lower PWA compared to its pre-stress baseline even after a 10-minute resting period. Therefore, it may be worthwhile to explore if stress tasks longer than 5-min in duration and/or greater intensity than an arithmetic challenge have long-lasting effects on PWA in future studies.

Our study further concluded that mental stress did not affect the ability of a blood vessel to respond to reactive hyperemia as measured by the PAT ratio. Although, this finding is in agreement with one previous study that used the PAT device [6] and two other studies that used the BAFMD technique[10, 25], larger studies are needed to verify this finding.

Our study is the first report that reported minute to minute changes in the PWA during and after the completion of a stress task. Many variables known to acutely influence endothelial function were controlled for in our study design such as standardized time of day and room temperature used during PAT testing, abstaining from alcoholic/caffeinated beverages on the day of testing, overnight fasting, and standardization of posture, and probe placement. It is important to acknowledge a few limitations of our study. First, due to the small sample size, estimates of changes in the PWA and the PAT ratio were less precise. Second, we did not assess the effects of different mental stress tasks such as public speaking task or word stroop test to verify consistency of our findings. Third, we also did not assess different time intervals post-completion of the stress task. Therefore, we could not examine the time interval required for PWA to return to its pre-stress baseline.

If the intervention under study is a stress-inducing procedure or if study protocol involves exposure to stressors other than the intervention, the post-intervention PWA may show a decrease from its baseline via sympathetic nervous system activation. Investigators should keep this in mind prior to designing studies that involve PWA as a primary outcome and perform PAT testing prior to performing stressful procedures. Larger studies are required to examine (1) if there are greater decreases in the PWA from pre to post-stress task in responders compared to non-responders, (2) and if such decreases remain persistent even after the stressful procedure is eliminated, and (3) if the response to stress varies with tasks different in duration and intensity of stress.

Tables

Table 1. Description of the Study Variables

Study Variable	Time of Measurement	Description
Baseline PWA_{pre}	1	PWA obtained at baseline prior to the administration of the mental stress task. This baseline PWA also served as the baseline for the first PAT test
Baseline PWA_{MS}	2	A 1-minute segment of PWA obtained that ends 20 seconds prior to the beginning of the stress task
$PWA_{MS\ 0\text{-}30\ sec}$	3	PWA obtained for each 30-sec segment after the beginning of the mental stress task up to 5 minutes
$PWA_{MS\ 30\ sec\text{-}1\ min}$	4	
$PWA_{MS\ 1\text{-}1.5\ min}$	5	
$PWA_{MS\ 1.5\text{-}2\ min}$	6	
$PWA_{MS\ 2\text{-}2.5\ min}$	7	
$PWA_{MS\ 2.5\text{-}3min}$	8	
$PWA_{MS\ 3\text{-}3.5\ min}$	9	
$PWA_{MS\ 3.5\text{-}4\ min}$	10	
$PWA_{MS\ 4\text{-}4.5\ min}$	11	
$PWA_{MS\ 4.5\text{-}5\ min}$	12	
Mean PWA_{MS}	Mean of 3-12	Average PWA during stress
Baseline PWA_{post}	13	PWA obtained after a 10-min resting interval following the mental stress task performance. This baseline PWA also served as the baseline for the second PAT test
Stress Ratio	NA	A ratio of PWA at time 3 to time 2
Pre-MST PAT Ratio	NA	PAT ratio obtained from the first PAT test prior to the mental stress task performance
Post-MST PAT Ratio	NA	PAT ratio obtained from the second PAT test after the mental stress task performance

Table 2. Baseline Characteristics of Study Subjects

Age (years)	28.3 ± 8.6
Gender (M:F)	5:4
Race	
White	7 (77.8%)
Asian	2 (22.2%)
Systolic Blood Pressure (mmHg)	123.0 ± 10.9
Diastolic Blood Pressure (mmHg)	77.7 ± 5.8
Weight (pounds)	158.9 ± 18.5
Body Mass Index (kg/m^2)	23.9 ± 2.1

Values represent mean ± SD unless otherwise specified.

Table 3. Changes in the Mean PWA During and After the Performance of the Mental Stress Task from the Baseline

PWA	Time of Measurement	Occlusion Arm (Mean ±SD)	Control Arm (Mean ±SD)
Baseline PWA_{pre}	1	737.9 ± 567.8	757.6 ± 542.4
Baseline PWA_{MS}	2	670.1 ± 490.0	624.5 ± 482.7
$PWA_{MS\ 0\text{-}30\ sec}$	3	480.7 ± 455.9	456.0 ± 423.5
$PWA_{MS\ 30\ sec\text{-}1\ min}$	4	520.3 ± 500.7	484.5 ± 457.6
$PWA_{MS\ 1\text{-}1.5\ min}$	5	509.1 ± 491.4	477.0 ± 450.0
$PWA_{MS\ 1.5\text{-}2\ min}$	6	537.0 ± 526.9	499.9 ± 476.7
$PWA_{MS\ 2\text{-}2.5\ min}$	7	540.0 ± 515.4	489.5 ± 474.4
$PWA_{MS\ 2.5\text{-}3min}$	8	533.0 ± 512.7	482.1 ± 482.6
$PWA_{MS\ 3\text{-}3.5\ min}$	9	527.9 ± 485.6	509.6 ± 448.1
$PWA_{MS\ 3.5\text{-}4\ min}$	10	526.8 ± 455.8	518.4 ± 412.1
$PWA_{MS\ 4\text{-}4.5\ min}$	11	539.0 ± 465.7	528.5 ± 421.4
$PWA_{MS\ 4.5\text{-}5\ min}$	12	562.8 ± 497.1	545.6 ± 441.5
Mean PWA_{MS}	Average (Time 3-12)	527.7 ± 487.4	499.1 ± 443.1
Baseline PWA_{post}	13	562.2 ± 504.1	597.8 ± 484.5

Table 4. Results from Repeated Measures ANOVA: Comparing the Changes in the PWA During and After the Performance of the Mental Stress Task with Baseline

Comparison of PWA during and after the MST with baseline	PWA of Occlusion Arm		PWA of Control Arm	
	Change in PWA Estimate (95% CI†)	p-value*	Change in PWA Estimate (95% CI)	p-value*
Time 2 vs Time 1	-67.8 (-123.6, -11.9)	0.02	-133.1 (-195.7, -70.5)	<0.0001
Time 3 vs Time 1	-257.2 (-335.9, -178.4)	<0.0001	-301.6 (-389.8, -213.5)	<0.0001
Time 4 vs Time 1	-217.6 (-313.7, -121.4)	<0.0001	-273.2 (-380.7, -165.7)	<0.0001
Time 5 vs Time 1	-228.8 (-339.4, -118.1)	<0.0001	-280.6 (-404.2, -157.0)	<0.0001
Time 6 vs Time 1	-200.8 (-324.1, -77.5)	0.002	-257.7 (-395.3, -120.2)	0.0003
Time 7 vs Time 1	-197.8 (-332.6, -63.2)	0.004	-268.1 (-418.2, -118.1)	0.0006
Time 8 vs Time 1	-204.9 (-349.9, -59.90)	0.006	-275.5 (-436.9, -114.2)	0.001
Time 9 vs Time 1	-209.9 (-364.5, -55.4)	0.008	-248.0 (-419.7, -76.2)	0.005
Time 10 vs Time 1	-211.1 (-374.5, -47.7)	0.01	-239.2 (-420.6, -57.8)	0.01
Time 11 vs Time 1	-198.9 (-370.6, -27.2)	0.02	-229.1 (-419.5, -38.7)	0.02
Time 12 vs Time 1	-175.0 (-354.5, 4.5)	0.06	-212.1 (-410.9, -13.2)	0.04
Time 13 vs Time 1	-175.6 (-362.6, 11.3)	0.07	-159.9 (-366.6, 46.9)	0.13
Overall p-value	<0.0001		<0.0001	

*For comparisons of PWA using a repeated measures ANOVA

† CI, Confidence Interval

Table 5. Classification of the study subjects into stress responder and non-responder groups

Subject	Baseline PWA_{MS} (Time 2)	PWA in the first 30 sec. during MST (Time 3)	Stress Ratio	Group
1	635.43	580.46	0.91	SNR
2	120.68	134.61	1.12	SNR
3	1652.47	1385.21	0.84	SNR
4	1151.47	1041.24	0.90	SNR
5	84.83	60.62	0.71	SR
6	499.28	125.38	0.25	SR
7	684.01	318.18	0.47	SR
8	741.75	466.84	0.63	SR
9	460.71	213.78	0.46	SR

SNR, Stress Non-responder
SR, Stress Responder
MST, Mental Stress Task
Stress ratios presented in this table were computed using the PWA of the occlusion arm.
Baseline $PWA_{MS,}$ A 1-minute segment of PWA obtained that ends 20 seconds prior to the beginning of the stress task

Table 6. Changes in the PWA of the occlusion arm during and after the performance of the MST by responder status

PWA/Stress Ratio	Time of measurement	Within-Group Comparison		Between-Group Comparison	
		Stress-Responder, N=5	Stress Non-Responder, N=4	Difference between Responders and Non-Responders	
		(Mean ± SD)	(Mean ± SD)	Estimate (95%CI)	p-value‡
Baseline PWA_{pre}	1	482.0 ± 200.7	1057.7 ± 748.7	-575.6 (-1732.0, 580.7)	0.22
Baseline PWA_{MS}	2	494.1 ± 257.9	890.0 ± 659.9	-395.9 (-1147.7, 355.9)	0.25
PWA during the first 30 sec of MST	3	237.0 ± 160.9	785.4 ± 544.9	-548.4 (-1385.9, 289.1)	0.14
Baseline PWA_{post}	13	316.6 ± 261.4	869.2 ± 600.2	-552.6 (-1250.3, 145.0)	0.10
Diff PWA (PWA_{post} - PWA_{pre})	Time 13-Time 1	-165.4 ± 236.7	-188.4 ± 328.4	23.0 (-420.7, 466.7)	0.91
p-value†		0.19	0.33	NA	NA
Stress Ratio	Time 3/Time 2	0.50 ± 0.18	0.94 ± 0.12	NA	NA

* CI, Confidence Interval
NA, not applicable
† For comparisons of PWA using a paired t-test
‡ For comparisons of PWA using an independent sample t-test

Table 7. Changes in the PWA of the control arm during and after the performance of the MST by responder status

PWA/Stress Ratio	Time of measurement	Within-Group Comparison		Between-Group Comparison	
		Stress-Responder, N=5	Stress Non-Responder, N=4	Difference between Responders and Non-Responders	
		(Mean ± SD)	(Mean ± SD)	Estimate (95%CI)	p-value‡
Baseline PWA_{pre}	1	513.4 ± 232.1	1062.9 ± 699.4	-549.5 (-1327.3, 228.2)	0.14
Baseline PWA_{MS}	2	469.8 ± 297.8	817.9 ± 642.9	-348.1 (-1105.2, 408.9)	0.31
PWA during the first 30 sec of MST	3	256.7 ± 210.6	705.0 ± 519.9	-448.3 (-1044.3, 147.7)	0.12
Baseline PWA_{post}	13	390.2 ± 314.3	857.2 ± 576.9	-467.0 (-1174.8, 240.7)	0.16
Diff PWA (PWA_{post} - PWA_{pre})	Time 13-Time 1	-123.2 ± 104.5	-205.7 ± 236.0	82.5 (-192.8, 357.7)	0.50
p-value†		0.06	0.18	NA	NA
Stress Ratio	Time 3/Time 2	0.57 ± 0.22	0.95 ± 0.17	NA	NA

* CI, Confidence Interval
† For comparisons of PWA using a paired t-test
‡ For comparisons of PWA using an independent sample t-test;
MST, Mental stress task
NA, not applicable

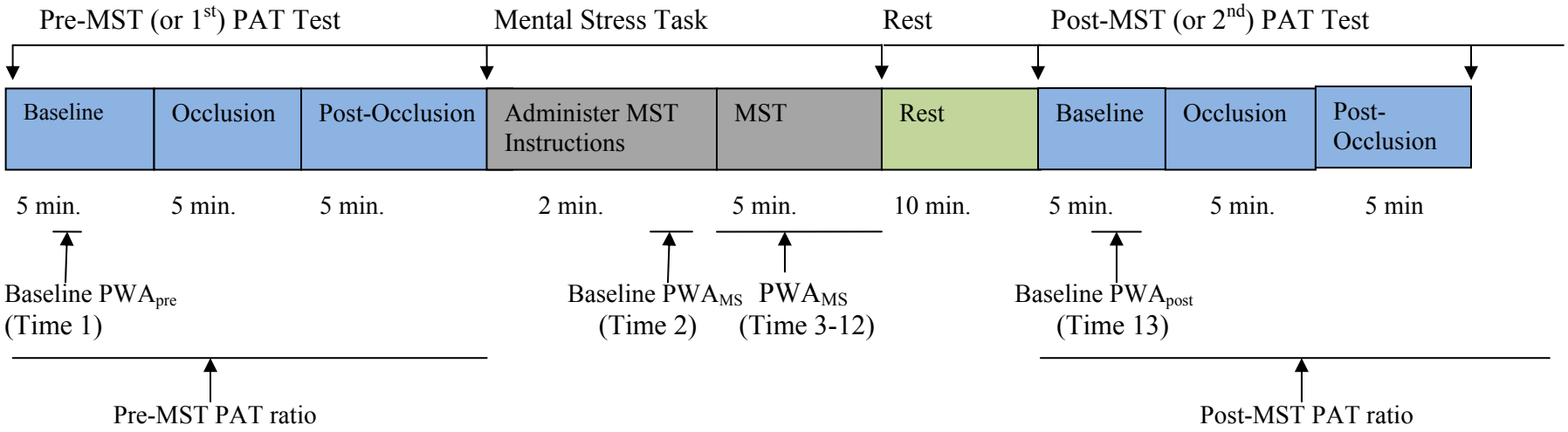
Table 8. Changes in PAT ratio: overall and by group

PAT Ratio	Overall	Within-Group Comparison		Between-Group Comparison	
	N=9	Stress Responder, N=5	Stress Non-responder, N=4	Difference between stress responder and non-responder	
Pre-MST PAT Ratio	1.63 ± 0.5	1.70 ± 0.7	1.54 ± 0.2	0.16 (-0.72, 1.04)	0.65
Post-MST PAT Ratio	1.79 ± 0.4	1.82 ± 0.5	1.75 ± 0.4	0.07 (-0.59, 0.74)	0.80
Diff (Post-Pre MST)	0.16 ± 0.5	0.12 ± 0.7	0.21 ± 0.2	-0.09 (-0.97, 0.80)	0.83
p-value†	0.39	0.72	0.17		

* CI, Confidence Interval
† For comparisons of PAT ratio using a paired t-test
‡ For comparisons of PAT ratio using an independent sample t-test

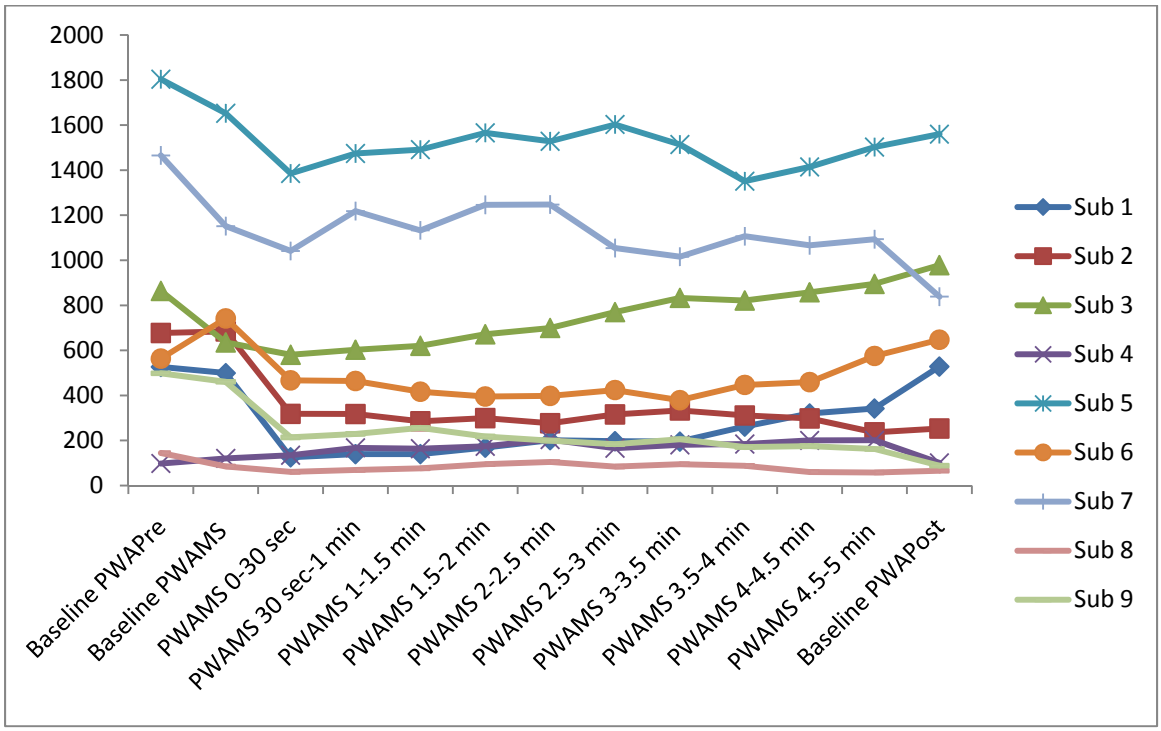
FIGURES

Figure 1. Experimental Timeline for the Study Protocol



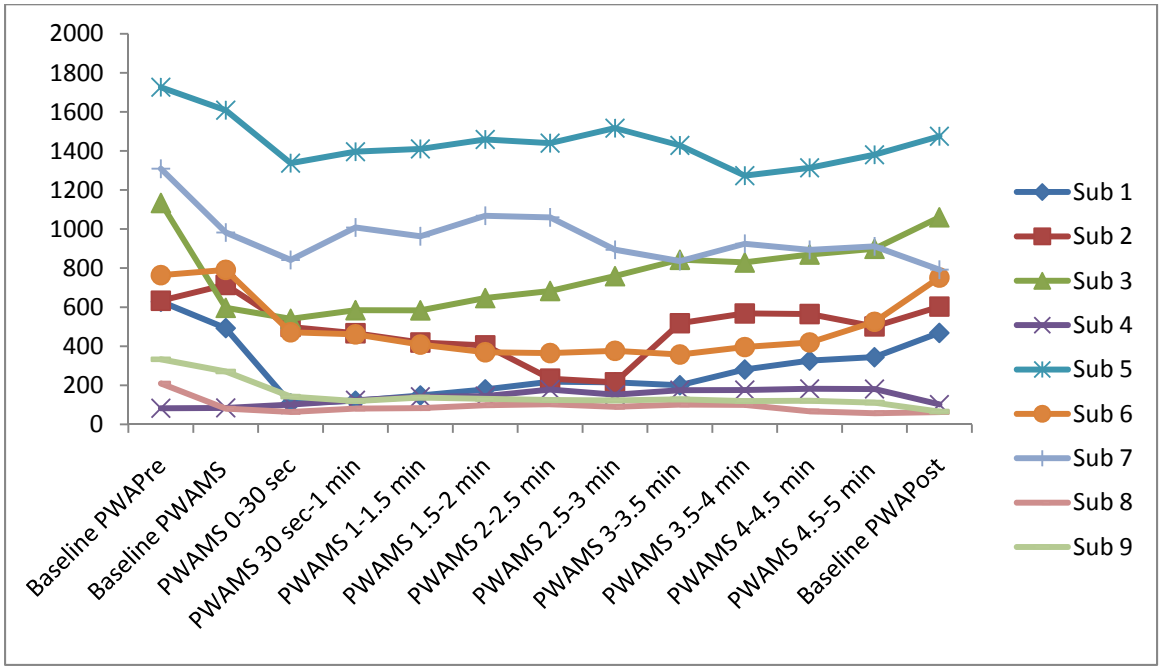
MST, Mental Stress Task
The entire study protocol was conducted in approximately 50 minutes.
The arrows and lines represent the time intervals during which the PWA and the PAT ratio measurements were obtained.

Figure 2. The changes in the PWA of the occlusion Arm during and after the mental stress task from its baseline



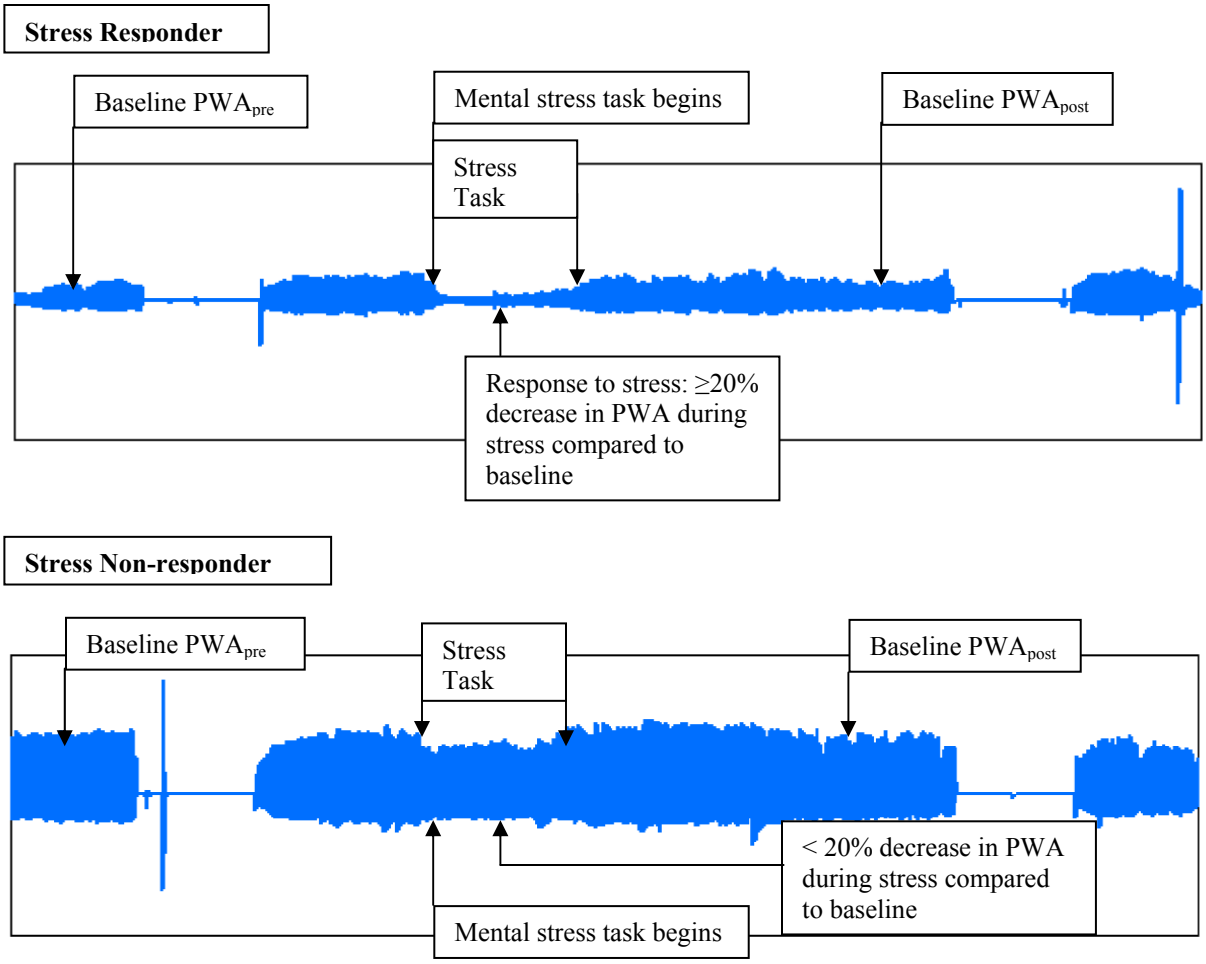
Each line represents an individual subject (Sub 1-Sub 9).

Figure 3. The changes in the PWA of the Control Arm during and after the performance of mental stress task from its baseline



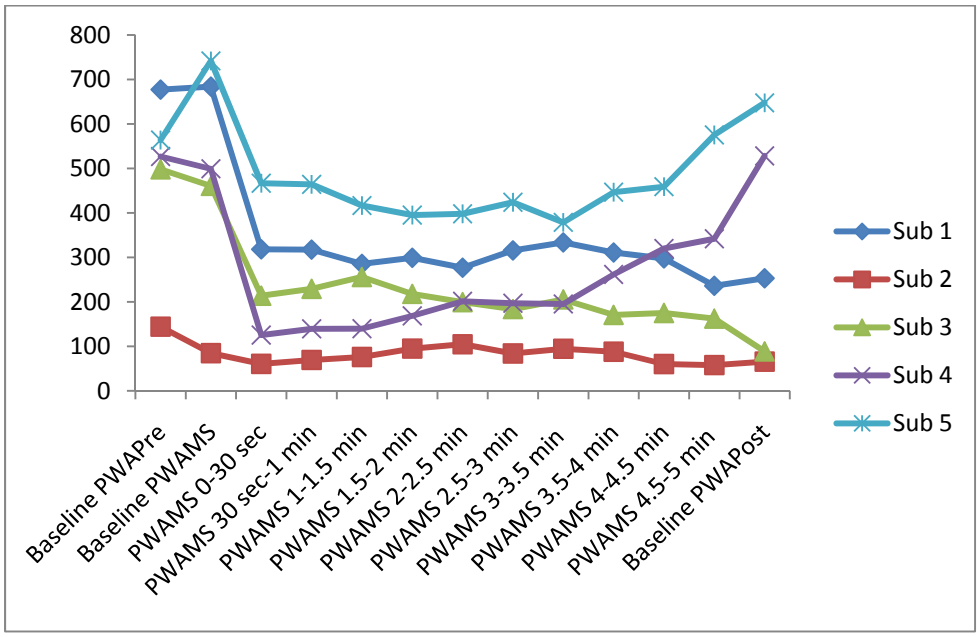
Each line represents an individual subject (Sub 1-Sub 9).

Figure 4. Pulse wave amplitude tracings from a stress responder and a non-responder



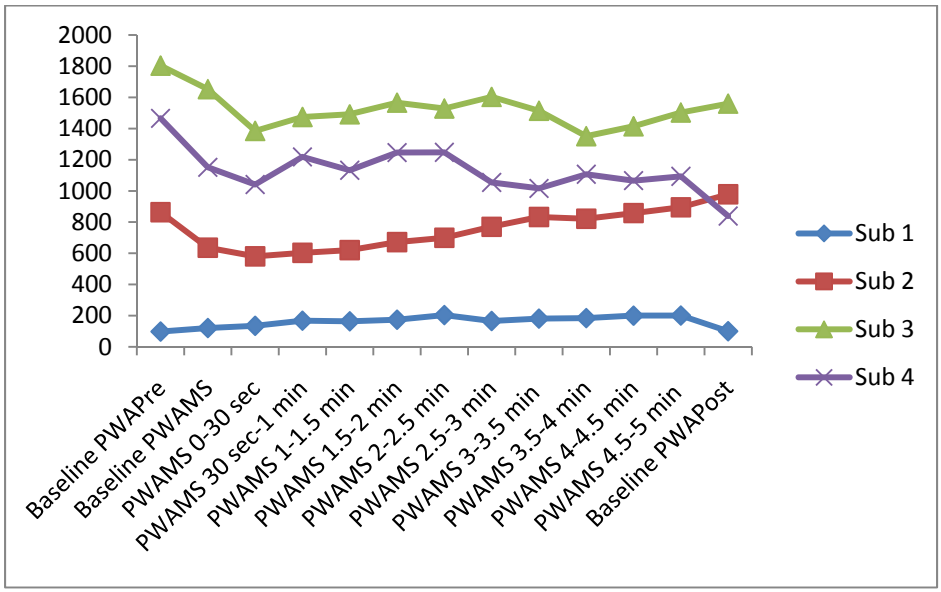
The top panel shows a PWA tracing from a stress responder with a ≥20% decrease in the PWA during stress from the baseline. The bottom panel shows a PWA tracing from a stress non-responder with a <20% decrease in the PWA during stress from the baseline.

Figure 5. The changes in the PWA of occlusion arm during and after the performance of mental stress task from its baseline among stress responders



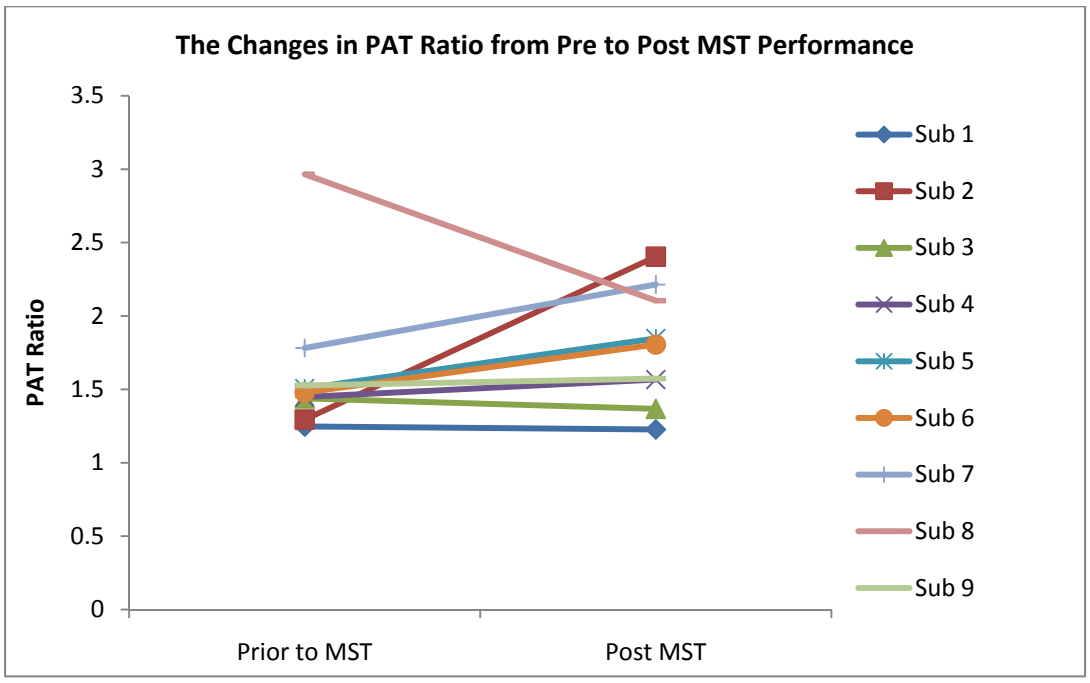
Each line represents an individual subject (Sub 1-Sub 5).

Figure 6. The changes in the PWA of occlusion arm during and after the performance of mental stress task from its baseline among stress non-responders



Each line represents an individual subject (Sub 1-Sub 4).

Figure 7. The changes in the PAT ratio after the performance of the mental stress task from its baseline



Each line represents an individual subject (Sub 1-Sub 9).

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THE EFFECTS OF A 2-HOUR EXPOSURE TO DIESEL EXHAUST AND SECONDARY ORGANIC AEROSOLS ON MARKERS OF ENDOTHELIAL FUNCTION: FINDINGS FROM A CONTROLLED EXPSOURE STUDY

by

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ABSTRACT OF MANUSCRIPT 2 OF 3

THE EFFECTS OF A 2-HOUR EXPOSURE TO DIESEL EXHAUST AND SECONDARY ORGANIC AEROSOLS ON MARKERS OF ENDOTHELIAL FUNCTION: FINDINGS FROM A CONTROLLED EXPSOURE STUDY

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ABSTRACT

Introduction: Previous epidemiologic studies have demonstrated that cardiovascular events may be triggered within a few hours of exposure to particulate matter. The role of endothelial dysfunction has been recently investigated in several epidemiologic and panel studies. While researchers have reported the use of brachial artery flow mediated dilation (BAFMD) in examining hyperacute effects of air pollutants, no studies have attempted to examine such effects using two new promising methods of measurement of endothelial function, namely the EndoPAT technology and plasma nitrite concentration.

Objectives: We examined the acute changes in the EndoPAT measurements, plasma nitrite concentration and BAFMD measurements following a 2-hour exposure to diesel exhaust (DE) and secondary organic aerosols (SOA) compared to clean air (CA) in a controlled environmental facility in young healthy volunteers.

Methods: Using a controlled exposure study design, data on endothelial function markers were collected from 52 healthy, non-smoking men and women between the ages of 18-30 years. Endothelial function was measured using three methods, i.e. BAFMD, EndoPAT device and plasma nitrite concentration prior and immediately after a 2-hour exposure session during which subjects were exposed to fresh DE (200 $\mu g/m^3$), SOA

(200 μg/m^3) or filtered CA in a random order. The effect of exposure to DE/SOA compared to CA on pre to post changes in six outcome measures was assessed using linear mixed models. The outcome variables were baseline pulse wave amplitude (PWA) and a PAT ratio obtained from the EndoPAT device, plasma nitrite concentration, resting brachial artery diameter (BAD), post-cuff deflation BAD and percent FMD obtained from the BAFMD technique.

Results: The plasma nitrite concentration showed a non-significant decrease following the exposure to DE and SOA compared to CA [DE versus CA: -6.9 nM; 95% CI: -26.4, 12.6, SOA versus CA: -13.8; 95% CI: -40.2, 12.7] The mean PAT ratios showed a significant increase following the exposure to both experimental aerosols and control CA [For example, pre to post DE: 1.66 ± 0.4 versus 1.87 ± 0.5; p=0.02] This increase was independent of the exposure condition and it may be attributed to a possible carry-over effect related to poor intra-day reproducibility of PAT tests, especially when tests are repeated within a short time period of 2 hours. The baseline PWA for both arms showed a significant decrease from pre to post-exposure to DE, SOA and CA indicating an increased vascular tone following the exposure sessions [For example, pre to post DE change in PWA of occlusion arm: 561.6 ± 427.5 versus 195.7 ± 166.9; p<0.0001] This decrease was lesser in magnitude on DE and SOA days compared to CA day, even after eliminating the effect of the order of visit. No substantial changes were observed in the resting BAD, post-cuff deflation BAD and %FMD. However, a trend towards vasoconstriction (decrease in the resting BAD) following exposure to DE was observed.

Conclusions: A decrease in the plasma nitrite concentration following the exposure to air pollutants is a promising finding. Although the magnitude of the decrease may not be

clinically appreciable, these results provide a basis for larger studies to confirm the finding. Also, future studies are warranted to examine the changes in plasma nitrite concentration following a longer time interval such as 6 or 12 hours post-exposure. In addition, the study also concluded that the EndoPAT device may not be a useful method to capture the acute changes in endothelial function in response to air pollutants within a short time frame.

THE EFFECTS OF A 2-HOUR EXPOSURE TO DIESEL EXHAUST AND SECONDARY ORGANIC AEROSOLS ON MARKERS OF ENDOTHELIAL FUNCTION: FINDINGS FROM A CONTROLLED EXPSOURE STUDY

Introduction

Endothelial dysfunction has been proposed as one of the operating mechanisms by which exposure to particulate matter (PM) can cause cardiovascular diseases (CVD). Endothelial dysfunction is defined as inability of blood vessels to dilate fully in response to an appropriate stimulus and it can arise under conditions of systemic inflammation and/or oxidative stress. It has been suggested that a brief exposure to air pollutants can affect vascular reactivity.

There is substantial evidence suggesting that cardiovascular events may be triggered within a few hours of exposure to high concentration of particles. A handful of studies have demonstrated that exposure to air pollution particles may exhibit hyperacute effects via direct interaction of nano-scale particles and soluble PM constituents with the cardiovascular system.[1, 2, 3, 4] For instance, Rich et al reported that the risk of ventricular arrhythmia and paroxysmal atrial fibrillation within a few hours prior to the onset of detectable arrhythmia episode was increased in association with exposure to ambient air pollutants.[2, 3] Another study reported that the risk of myocardial infarction (MI) increased by 48% for a 25 $\mu g/m^3$ increase in $PM_{2.5}$ over the preceding 2 hours indicating a potential role of exposure to $PM_{2.5}$ in triggering of MI.[4]

Furthermore, recent studies have shown the hyperacute effects of exposure to particles on endothelial function measured using a physiological method, namely,

flow-mediated dilation of the brachial artery (BAFMD) in healthy adults. Two studies demonstrated a significant brachial artery vasoconstriction response following a 2-hour inhalation of mixture of ambient fine particles and ozone or diesel exhaust among healthy adults in a controlled exposure study.[5, 6] Another study by Schneider et al (2008) reported a decrease in BAFMD in association with $PM_{2.5}$ within the first 24 hours suggesting acute endothelial dysfunction among diabetic individuals.[7] To date, the utility of non-invasive methods of measurement of endothelial function other than BAFMD has not been investigated to demonstrate the effects of exposure to air pollution particles on endothelial function.

Two promising methods of measurement of endothelial function, namely peripheral arterial tonometry using the EndoPAT device and plasma nitrite assay, have been utilized as a diagnostic tool in several experiments to identify individuals with endothelial dysfunction. While the EndoPAT technique is similar to the BAFMD technique in measuring a physiological response of a blood vessel to a hyperemic stimulus, plasma nitrite assay is a biochemical method of measuring nitric oxide availability. The EndoPAT device has been employed to determine if vascular reactivity is altered in certain conditions such as diabetes mellitus, CVD, polycystic ovarian disease, and depression.[8, 9, 10, 11, 12, 13, 14, 15] It has been utilized to detect abnormal coronary vascular function among individuals with CVD.[16, 17] The utility of the PAT device has also been established in settings where short-term interventions or treatments have been shown to produce potential beneficial or worsening effects on endothelial function. However, no studies have reported the use of this device to examine the acute effects of air pollution particles on endothelial function.

There is substantial evidence that plasma nitrite level is a biochemical marker of endothelial dysfunction. Kleinbongard et al and Kehmeier et al examined whether plasma nitrite concentration are reduced in patients with endothelial dysfunction and additionally, Kleinbongard et al examined if the decrease in nitrite is correlated with the number of cardiovascular risk factors.[18, 19] They concluded that plasma nitrite concentration decreased progressively with increasing number of cardiovascular risk factors. They also found decreasing levels of nitrite in patients with established endothelial dysfunction i.e. patients with CVD.

A graded exercise test was recently used by Allen et al to stimulate nitrite levels in 4 groups of patients with increasing severity of endothelial dysfunction.[20] These groups were as follows: (1) risk factors, but no vascular disease, (2) type II diabetes mellitus (DM) with no vascular disease, (3) diagnosed peripheral arterial disease (PAD), and (4) DM + PAD. Following the exercise test, plasma nitrite was shown to increase in the group with risk factors, no change in the DM group, and a decrease in the PAD and PAD + DM group. This study demonstrated the utility of plasma nitrite measurement as a novel biochemical marker for differentiating patients with varying degrees of endothelial dysfunction. Only one study has reported its use in an environmental experiment demonstrating a significant decrease in plasma nitrate levels, not nitrite following a brief exposure to elemental carbon ultra-fine particles.[21]

To explore the utility of these physiological and biochemical markers in explaining the mechanistic link between air pollution and endothelial dysfunction, we sought to examine the acute changes in endothelial function using the established technique of BAFMD as well as newer techniques such as the EndoPAT measurements

and plasma nitrite concentration following a 2-hour exposure to diesel exhaust and secondary organic aerosols in young healthy volunteers. The specific aims of this study were to examine: (1) the acute changes in EndoPAT, (2) plasma nitrite, and (3) BAFMD measurements following a 2-hour exposure to fresh diesel exhaust (200 $\mu g/m^3$) and secondary organic aerosols (200 $\mu g/m^3$) in young healthy volunteers.

Materials and Methods

Data Source

A double-blind crossover experiment using controlled exposure conditions was conducted at the Clinical Center of the Environmental and Occupational Health Sciences Institute (EOHSI) located on the Rutgers University campus in Piscataway, New Jersey. The purpose of this study was to examine the effects of 2-hour exposure to two kinds of air pollution particles, namely diesel exhaust (DE) and secondary organic aerosols (SOA) on platelet activation markers and endothelial function in a group of healthy, non-smoking young volunteers. Data on both outcomes were also collected following a 2-hour exposure to filtered clean air (CA) as control. The project was funded by the United States Environmental Protection Agency. The study was approved by the University of Medicine and Dentistry of New Jersey –Robert Wood Johnson Medical School Institutional Review Board.

Subject Recruitment and Clinical Evaluation

Healthy, non-smoking men and women between the ages of 18-30 years were recruited from the Rutgers University community using advertisement flyers between December

2005 and April 2009. Potential candidates were screened by an experienced research staff using a standard telephone questionnaire to determine their eligibility for participation. Candidates were excluded if they indicated presence of cardiovascular disease, cancer, stroke, hypertension, pulmonary diseases including asthma, and kidney/liver disease during the screening process. Eighty-two candidates who passed the initial telephone screening were further scheduled to complete a history and physical examination under a study physician's supervision. The physical examinations were conducted at the EOHSI Clinical Center. All subjects gave informed consent at this appointment. The purpose of the evaluation was to rule out presence of any medical conditions that would preclude study participation. Subjects underwent a vital examination, standard laboratory evaluation (complete blood count with differential and platelet count), measurement of blood pressure, an electrocardiogram, lung function test and a medical examination by a physician. In addition, subjects were asked to complete a medical questionnaire that asked history of medical illnesses and prior as well as current use of medications. No clinically significant abnormalities were allowed to be present. Exclusion criteria were: history of neurologic disease, stroke or cardiovascular disease, pulmonary disease including asthma, gastrointestinal disorders, known endocrine disease, major psychiatric conditions, pregnant or lactating women, and females using any form of hormonal contraception such as oral contraceptive pills or patch. Medical records obtained from the physical examination were reviewed and evaluated by the study physician to ascertain eligibility. Based on the physician's review, 72 individuals were invited to participate in the study. For safety and comfort reasons, 10 individuals were declined participation. During this visit, subjects were familiarized with a controlled environmental facility

(CEF) located on the third floor of the EOHSI and briefly explained experimental procedures involved with study participation.

Scheduling of Exposure Sessions

Following medical clearance, 72 subjects were scheduled for three exposure sessions conducted in the morning and three follow-up visits conducted in the afternoon on the same day. The actual study participation was completed within two months of subjects' physical exam appointment in most cases. Each subject was tested one day per week over 3 consecutive weeks. For the most part, all visits were conducted at least one week apart. Subjects were randomly assigned to receive three exposure conditions: DE, SOA and CA. Both subjects and research staff conducting the experiment were blinded to the order of exposure. An additional 9 subjects dropped out for personal or scheduling reasons prior to commencing exposures, reducing the sample size to 63 subjects.

Availability of Data

Since evaluation of endothelial function using the EndoPAT tonometer, BAFMD and plasma nitrite assay required purchasing of new equipment, hiring and training of staff and conducting several validation experiments to generate accurate data, these tests were added to the experimental design at different time intervals after initiation of the primary study. Thus, of the 63 subjects enrolled in the primary study, it was possible to obtain data on EndoPAT, plasma nitrite concentration and BAFMD from 52, 49 and 18 subjects, respectively. All outcomes including assessment of endothelial function were evaluated at baseline, i.e. before exposure to air pollutants and immediately following a 2-hour

exposure to DE, SOA and to CA. Table 1 shows the time points when endothelial function was measured for each subject. Availability of complete data on endothelial function markers at all exposure time-points was further reduced due to the following reasons: poor brachial artery image quality, poor PAT signals, poor quality of blood aliquots and missing blood samples at certain time-points due to subject unavailability or difficulty in obtaining blood samples. Table 2 provides a summary of available data on each outcome for DE/CA and SOA/CA comparisons. It may also be noted that the reduced number of subjects receiving the SOA exposure was due to logistical constraints in scheduling exposure sessions, as well as an a priori preference for assessing the effects of DE over the SOA when only two sessions could be scheduled for a subject.

Study Protocol

Subjects were asked to arrive following an overnight fast beginning at midnight the day before testing. They were instructed to abstain from any caffeinated and/or alcoholic beverages as well as vigorous exercise 12 hours prior to the testing. Subjects were also instructed to abstain from any aspirin containing medications 2 weeks prior to the testing to avoid their effect on platelet activation markers. On the day of each experimental session, subjects reported to the Clinical Center at 8:00 A.M. and a research staff performed a check-in to ascertain that the above conditions have been met. A pregnancy test was administered to female subjects. A pre-exposure blood sample was collected into vacuum tubes and aliquotted for measurement of plasma nitrite and other biomarkers of inflammation. Then endothelial function was measured using the two methods/devices simultaneously i.e. the EndoPAT tonometer and the BAFMD. The measurement of

endothelial function was performed approximately between 8:30 A.M and 9:00 A.M. on the day of testing. A detailed description of the methods used for data collection and the study variables has been provided in Appendix of the dissertation. Subjects were then taken to the CEF and seated comfortably in a chair. They were allowed to read or study, but physical activity was limited because of the potential effect on vascular reactivity and other outcomes. Subjects were allowed to use a restroom during the exposure session. Subjects were exposed for approximately 2 hours to either DE (200 $\mu g/m^3$), SOA (200 $\mu g/m^3$), or CA in a random order. At the end of the 2-hour exposure session, another blood sample was obtained for measurement of the same parameters mentioned above. The endothelial function measurement using both methods was repeated thereafter. The measurement of endothelial function following the completion of the exposure session was performed between 12:00 P.M. and 12.30 P.M.

Controlled Environmental Facility (CEF)

The CEF located on the third floor of the EOHSI is a large stainless steel chamber in which conditions of temperature and humidity are controlled. The chamber dimension is 7.3 ft. high by 13.5 ft. wide by 9 ft. deep for a volume of 887 cubic ft. (or 25 m^3). The chamber is interfaced to maintain constant environmental (exposure) conditions for human health effects studies. It has tables, chairs, a personal computer, and a lavatory for subject use during experiments.

Exposure Conditions

A. Diesel Exhaust: All study exposures were conducted in the CEF. The temperature and relative humidity were 70ºF±SD and 35%±SD, respectively during all exposure sessions. The DE was generated by a 5500W electricity generator (Yanmar, Model YDG 5500EE) that contains a 406 cc displacement air-cooled engine. The engine was operated using Number 2 undyed low sulfur on-highway fuel and 40 weight motor lube oil. Sufficient fuel to complete the study was purchased at the beginning of the project to eliminate variation in fuel composition throughout the study. The engine, loaded with several space heaters, was maintained at 100% of rated capacity during each experimental session. The engine was located 2 floors above the ceiling of the CEF, in the penthouse of the EOHSI Building. The dilution/delivery system included two single-stage mass reduction devices. The first-stage device was a 10 position butterfly valve that divided diesel exhaust between the waste exhaust pipe and the second-stage mass reduction device, a variable-speed blower. After the two mass reductions, the desired amount of diesel exhaust was introduced into the CEF air delivery stream to achieve the targeted DE particulate matter concentration (200$\mu g/m^3$) within the CEF. The CEF air delivery stream was filtered through a series of HEPA filters and activated carbon cartridges to remove ambient particles and gaseous pollutants.

B. Clean Air: Ambient air was drawn into the CEF through coarse and High-Efficiency Particulate Air (HEPA) filters, then filtered across activated carbon to remove ozone and organics.

C. Secondary Organic Aerosol: The SOA generation system used gas-phase reactions between ozone and d-limonene in a large glass reactor. Spectrometry analyses have identified various products of reactions between ozone and terpenes. [22] Generally, ozone

attacks the >C=C< bond of the limonene and forms a primary ozonide, which rapidly decomposes to carbonyls and Criegee biradicals. Criegee biradicals react further to form limonic acid, limonaldehyde, limononic acid, etc. These products have low vapor pressure and undergo gas-to-particle portioning, resulting in the formation of SOA. The generated SOA was carried by a heated clean air stream at a flow rate of 10 LPM and delivered to the CEF.

D. Exposure System Stability: All exposure systems were optimized to achieve stable concentrations in the CEF (Table 3). Concentrations of $PM_{2.5}$, nitrogen oxides (NOx), and carbon monoxide (CO) were monitored throughout each DE, SOA or CA session. Real-time PM2.5 mass concentration was measured using a SidePak Aerosol Monitor Model 8520 (TSI Inc., Shoreview, MN) calibrated with the same diluted DE or SOA in the CEF. Particle number concentration for the size range of 0.01 – 1 μm was measured with a Condensation Particle Counter (Model 3007, TSI, Inc.). NOx was monitored using a chemiluminescent NOx monitor (Thermo Electron Corp. Franklin, MA). CO was monitored using a Langan Model T15v CO monitor (Langan Products Inc, San Francisco, CA). Average concentrations for specific pollutants over the course of the study are shown in Table 3. The average total particle number concentration for SOA, measured with a Condensation Particle Counter (TSI, Model 3007), was 16,076 +/- 5,233 particles/cm^3, and average mass was 193.8 +/- 10.7 $\mu g/m^3$. For DE, average values were 72,717 +/- 36053 particles/cm^3 and 192.7 +/-7.2 $\mu g/m^3$, while for CA they were 3,460 +/- 1,963 particles/cm^3 and 4.8 +/- 3.9 $\mu g/m^3$.

Study Variables

On each visit, baseline pulse wave amplitude (PWA) for the occlusion and the control arm and a PAT ratio were obtained from the PAT device before and after the exposure to DE, SOA and CA. At each of these time points, plasma nitrite concentration was also measured. Using the BAFMD technique, three variables were obtained before and after the exposure to DE and CA: (1) resting brachial artery diameter (BAD), (2) post-cuff deflation BAD obtained at 60 seconds after BP cuff deflation, and (3) flow-mediated dilation (FMD%) at 60 seconds calculated as percent difference in post-cuff deflation diameter at 60 seconds from the resting BAD. We examined the effect of exposures in the original dataset (N=14) as well as two subsets of the original data set (N=12 and N=8 respectively) due to lack of optimal studies. In the first subset (N=12), data from two subjects were excluded: (1) if the smallest and the largest resting BAD measurements showed a greater than a 10% change or (2) if the change in FMD from 30 to 60 seconds was either too much or too small. In the second subset (N=8), an additional 4 subjects were excluded due to unavailability of complete brachial artery flow data.

Statistical Analyses

Statistical analysis was conducted for each outcome variable separately. A basic review (means, standard deviations, distributions, boxplots/histograms) of each outcome variable at each exposure time-point (i.e. pre/post-exposure DE, pre/post-exposure CA and pre/post-exposure SOA) was conducted. Subjects with missing data at any exposure time point were excluded from analysis.

The effects of DE and SOA on each outcome were examined separately. For each subject visit, the pre-exposure value in each outcome variable (i.e. PAT ratio, baseline PWA, nitrite, resting BAD, post-cuff deflation BAD and FMD) was subtracted from the post-exposure value, to estimate the change (or delta) in each outcome associated with a particular exposure session (CA, SOA or DE). A mean and standard deviation of these changes or deltas were reported. A paired t-test was conducted to examine if the two measurements (i.e. pre and post-exposure) were different from one another.

Next, the change or delta calculated for CA day, was subtracted from the delta calculated for DE day, resulting in a grand delta $_{(DE-CA)}$ for each subject. This was repeated for SOA to generate a grand delta $_{(SOA-CA)}$ for each subject. This calculation provided a control for the effects of the experiment beyond the exposure. A mean grand delta for DE and a mean grand delta for SOA were then calculated to estimate an average change in each outcome measure associated with each exposure condition across all study subjects. Next, a mixed linear model (Model 1) with F-tests and a random intercept to assess the effects of exposure (DE, SOA or combined exposure to DE or SOA) on pre to post changes in each outcome measure was used after accounting for repeated measurements within subjects/sessions and the order of visit (first versus second or third). Two additional sensitivity analyses were conducted: (1) a mixed linear model (Model 2) was fit to examine the effects of exposure (DE, SOA or combined exposure to DE or SOA) on the post-exposure measurement after adjusting for the pre-exposure measurement and the order of visit. This approach was used as it accounts for regression to the mean and does not force a relationship between pre and post measurements, (2) In

order to eliminate the effect of the order of visit, subjects with either DE or CA exposure on their first visit were excluded for DE/CA comparison and subjects with either SOA or CA exposure on their first visit were excluded for SOA/CA comparison. After excluding those, data from 10 and 11 subjects were used for DE/CA and SOA/CA comparison, respectively. At each stage of the analyses, diagnostics were conducted, including tests for normality and homogeneity of variances between groups. Statistical significance was set at $p<0.05$. SAS software version 9.1.3 (©, SAS Institute Inc., Cary, North Carolina) was used for all analyses.

Results

Baseline characteristics of Study Subjects

Table 4 shows demographic and baseline clinical characteristics of the study subjects. The subjects were healthy university students, predominantly white males. Clinical characteristics such as blood pressure, body mass index, and lipid profile measurements were in the normal range.

Changes in EndoPAT Measurements

Table 5 shows the mean changes in the PAT measurements following the exposure to DE, SOA and CA. The mean PAT ratio showed a significant increase following the exposure to DE (1.66 ± 0.4 versus 1.87 ± 0.5; $p=0.02$). The mean PAT ratio also showed an increase following the exposure to SOA, the change was not statistically significant (1.68 ± 0.5 versus 1.87 ± 0.5; $p=0.08$). For both comparisons, the mean PAT ratio showed a significant increase following the CA exposure ($p=0.004$, $p=0.009$ for DE and

SOA comparison, respectively). The baseline PWA for both arms showed a significant decrease from pre to post-exposure to DE, SOA and CA (for example: pre to post DE change in PWA of occlusion arm: 561.6 ± 427.5 versus 195.7 ± 166.9; $p< 0.0001$) This finding suggested an increase in the vascular tone following the exposure session, regardless of the exposure condition (i.e. DE, SOA, or CA).

The effect of the exposure to DE or SOA compared to CA on pre to post-exposure change in the PAT measurements was examined using mixed linear models. The results from the linear mixed models (Model 1) are shown in Table 6. Compared to CA, the exposure to DE and SOA produced a lesser increase in the PAT ratio (DE versus CA: -0.02; 95% CI -0.23, 0.19, p=0.86). When both exposures i.e. DE and SOA were combined, the exposure to either of the air pollutant still produced a lesser increase in the PAT ratio. Compared to CA, the exposure to DE or SOA produced a lesser decrease in baseline PWA for both arms, with a significantly lesser decrease observed with SOA (SOA versus CA for PWA of occlusion arm: 118.8; 95% CI: 23.3, 214.2; p=0.02). The combined exposure to DE and SOA also produced a lesser decrease in baseline PWA for both arms. The effect of exposure to DE or SOA on the post-exposure PAT measurements (Model 2) was examined using mixed linear models (Table 7). The post-DE or SOA exposure PAT ratio was lower compared to the post-CA exposure PAT ratio. The post-DE or SOA exposure baseline PWA was higher compared to the post-CA exposure PWA for both arms. When the effects of exposure were examined in a subset of 10 and 11 subjects after excluding their first visit as a part of sensitivity analyses (Table 8), the effect estimates did not differ much and remained comparable to the previous results shown in Table 6.

Changes in Resting Brachial Artery Diameter

Table 9 shows the changes in the mean resting BAD following the exposure to DE and CA in the original and the two subsets of the data. Overall, the mean resting BAD showed a tendency towards a decrease from pre to post-exposure to DE as well as CA (for example pre to post DE change in original data set: 3.77 ± 0.5 versus 3.73 ± 0.4; p=0.42). Although, none of these changes were statistically significant, the decrease in the resting BAD indicated a trend towards vasoconstriction and an increased vascular tone regardless of the exposure condition (i.e. DE or CA).

The effect of exposure to DE on pre to post-exposure change in the resting BAD compared to CA was examined using mixed linear models (Table 10). Exposure to DE produced a greater decrease in the resting BAD compared to CA in both subsets 1 and 2. However, exposure to DE produced a lesser decrease in the resting BAD in the original data set. The effect of exposure to DE on the post-exposure resting BAD was examined using mixed linear models (Table 10). The post-DE exposure resting BAD tended to be higher in the original data set and lower compared to the post-CA exposure resting BAD in both subsets. None of these changes in the resting BAD achieved statistical significance.

Changes in Post-cuff deflation Brachial Artery Diameter

Table 11 shows the changes in the mean post-cuff deflation BAD following the exposure to DE and CA in the original and the two subsets of the data. Overall, the mean post-cuff

BAD showed minimal changes following both exposures. The effect of exposure to DE on pre to post-exposure change in the post-cuff deflation BAD compared to CA was examined using mixed linear models (Table 12). Exposure to DE produced a greater increase in the post-cuff deflation BAD compared to CA in the original data and subset 1 whereas a greater decrease in subset 2. The effect of exposure to DE on post-exposure resting BAD was examined using mixed linear models (Table 12). The post-DE exposure post-cuff deflation BAD tended to be higher compared to the post-CA exposure post-cuff deflation BAD in the original data and subset 1 whereas it tended to be lower in subset 2. None of these changes in the post-cuff deflation BAD were statistically significant.

Changes in Percent FMD

Table 13 shows the changes in the mean percent FMD following the exposure to DE and CA in the original and the two subsets of the data. Overall, the mean %FMD increased following both exposures in the original as well as both subsets except on the CA day in subset 1.

The effect of exposure to DE on pre to post-exposure change in the %FMD compared to CA was examined using mixed linear models (Table 14). Exposure to DE produced a greater increase in the %FMD compared to CA in the original data and both subsets. When the effect of exposure to DE on post-exposure %FMD was examined using mixed linear models (Table 14), it was observed that the post-DE exposure %FMD was lower compared to the post-CA exposure FMD in the original data and both subsets. None of these changes in the %FMD were statistically significant.

Changes in Plasma nitrite concentration

Table 15 shows the changes in the mean plasma nitrite concentration following the exposure to DE, SOA and CA. Plasma nitrite concentration showed a statistically non-significant decrease following the exposure to DE and SOA whereas it showed a non-significant increase following the exposure to CA (For example change from pre to post DE: 131.3 ± 69.9 versus 127.3 ± 58.4; p=0.65). The effect of exposure to DE or SOA on pre to post-exposure change in the plasma nitrite concentration compared to CA was examined using mixed linear models (Table 16). Compared to CA, the exposure to DE and SOA produced a greater decrease in the plasma nitrite concentration (for DE versus CA: -6.9; 95% CI: -26.4, 12.6; p=0.48). When both exposures i.e. DE and SOA were combined, the exposure to either of the air pollutant also produced similar results. The effect of exposure to DE or SOA on the post-exposure plasma nitrite concentration was examined using mixed linear models (Table 16). The post-DE or SOA exposure plasma nitrite level was lower compared to the post-CA exposure plasma nitrite level, however none of these effects were significant (Post DE versus post CA: -5.6; 95% CI: -23.2, 12.0; p=0.52).

Discussion

In the controlled exposure study conducted to examine the acute changes following 2 hour exposure to diesel exhaust and secondary organic aerosols, we found that: (1) the increase observed in the PAT ratio was independent of exposure condition and it may be attributed to a possible carry-over effect related to poor intra-day reproducibility of PAT

tests, especially when tests are repeated within a short time period of 2 hours, (2) the baseline PWA for both arms showed a significant decrease from pre to post-exposure to DE, SOA and CA indicating an increased vascular tone following the exposure sessions. This decrease was lesser in magnitude on DE and SOA days compared to CA day, even after eliminating the effect of the order of visit, (3) no significant and clinically meaningful changes were observed in the resting and post-cuff deflation BAD as well as FMD. The changes were largely dependent on the quality of individual tests. However, a trend towards vasoconstriction (decrease in the resting BAD) following exposure to DE was observed when the smallest subset (subset 2) of the original data was examined, and (4) the plasma nitrite concentration showed a non-significant decrease following individual or combined exposure to DE and SOA compared to CA.

Although not statistically significant, a decrease in plasma nitrite following the exposure to DE and SOA is a promising finding of the study. Although the magnitude of the decrease may not be clinically appreciable, these results not only provide a basis for larger studies to confirm the finding, but also establish the utility of plasma nitrite as a reasonable marker of endothelial function. A shortcoming of the study, however, was that data on plasma nitrite concentration following a hyperemic stimulus could not be obtained due to logistical constraints and limited laboratory resources. Due to this reason, we failed to compare the effects of exposure to DE/SOA on the change in nitric oxide production following a hyperemic stimulus, an outcome that is directly comparable to the changes in the PAT ratio and the percent FMD.

We observed that the peripheral vascular tone increased significantly (or baseline PWA decreased) following the exposure regardless of the exposure condition.

When the changes in the baseline PWA from pre to post-exposure were examined by the order of visit, the subjects also exhibited the lowest PWA on their first visit irrespective of the exposure condition. This finding can be partly explained by the fact that the anxiety and/or stress associated with the first visit may cause an increased vascular tone or lower PWA in individuals as reported earlier in the manuscript one/part two of this dissertation. When a grand delta was modeled to examine the effect of exposure on the change in baseline PWA, exposure to SOA and DE produced a lesser decrease in the baseline PWA compared to CA. This finding may be explained with two possibilities: (1) the baseline PWA measured prior to the DE/SOA exposure was lower compared to the baseline PWA measured prior to CA exposure and the post-DE/SOA exposure PWA was similar to the post-CA exposure PWA. Due to the differences observed in the baseline PWA prior to the beginning of the DE/SOA and CA exposures, the decrease associated with DE/SOA was lesser in comparison to CA, with a significantly lesser decrease observed with the exposure to SOA. It is somewhat surprising to observe different baseline (pre-exposure) values for the baseline PWA. Furthermore, this finding may not be attributed to the effect of order of visit as there were comparable numbers of subjects who received DE/SOA as their first visit and CA as their first visit and similar results were also obtained after subject's first visit was eliminated, (2) since the effects were observed in both arms consistently, the significantly lesser decrease associated with the SOA exposure may not be due to a local endothelial response, but instead a central or neurohumoral effect on the vascular tone. The exposure to SOA may reduce the central response and hence, the decrease on the SOA day may be lesser compared to CA.

We observed an increase in the mean PAT ratio following exposure to both experimental aerosols and control CA. These data may not necessarily suggest an effect of exposure to aerosols on the PAT ratio as (1) the changes were observed irrespective of the exposure condition, and (2) there were no significant changes in the PAT ratio due to the exposure to DE/SOA compared to CA. Furthermore, these data appear to indicate a possibility of a carry-over effect when PAT tests are repeated within a short time interval on the same day. Till date, only one study conducted by Liu et al has reported that PAT tests are reproducible within 1 and 2 hours after a baseline measurement. [23] However, in their experiment, a significant trend towards an increase in the PAT ratio was observed when tests were repeated 1/2 hour apart. In order to gain an understanding as to whether PAT tests are reproducible on the same day, we conducted a pilot study and the results from this study were presented in the manuscript one/part one. Based on those findings, we concluded that the PAT device does not produce reliable PAT ratio estimates when tests are repeated 2.5 hours apart in young and healthy population. The exposure study data not only confirmed the pilot study findings, but also raised questions about the technique's utility in investigating acute changes in endothelial function occurring in a short time interval.

In our study, we failed to observe significant changes in the resting BAD following the exposures, but the decrease observed following both DE and CA was consistent with the decrease observed in the baseline PWA following both exposures. A greater non-significant decrease in the resting BAD following the exposure to DE compared to CA observed in the two subsets of the original data indicated vasoconstriction of the brachial artery and this finding is in agreement with two previous

reports.[5, 6] However, the estimates presented in our study were largely affected by a small sample size and suboptimal BAFMD studies. Therefore, no conclusions can be made based on these data.

Previous studies that measured percent FMD (%FMD) as a marker of endothelial function following exposure to air pollutants have been inconclusive. Brook et al (2011) observed no relationship between BAFMD and community or personal level $PM_{2.5}$.[24] However, in an experimental setting, they found that %FMD increased non-significantly post-exposure to ambient fine particles and ozone.[5] Due to lack of a pre-exposure %FMD measurement, Peretz et al (2008) could not make inferences on pre to post exposure change in %FMD, however, they reported that the post-DE exposure %FMD was greater compared to the post-CA exposure. [6] The only study that showed a decrease in %FMD in association with particulate matter less than 2.5 micrometers ($PM_{2.5}$) during the first 24 hours was conducted in diabetic individuals and it examined the effect of ambient $PM_{2.5}$ levels on %FMD rather than an experimental exposure.[7] In our study, FMD showed a greater increase following exposure to DE compared to CA regardless of the data set used for calculation. We speculate that this greater increase on the DE day may be due to: (1) the differences in shear rates or stimulation of brachial artery at pre and post-exposure time points on DE and CA days, and (2) the fact that %FMD is inversely related with resting BAD. Since we observed a greater decrease in the resting BAD and almost no change in the post-cuff deflation BAD following the exposure to DE compared to CA, the %FMD increased by a greater magnitude on the DE day. It is important to acknowledge a number of challenges experienced during collection of BAFMD data. First, we could not obtain data on an originally planned number of

subjects due to logistical constraints such as unavailability of an experienced technician, change of data acquisition technique to improve data quality, and logistical challenges in scheduling subjects. In order to detect a significant change of 0.11 mm in resting BAD, we would be required to obtain pre and post exposure data from 23 subjects with 90% power and type I error rate of 0.05. However, due to the reasons listed above, the target number was not achieved. Second, the quality of our data was not optimal mainly due to inexperience and inadequate training of the vascular technician who performed these studies. For instance, of the 119 individual studies performed on 29 subjects, only 47% (56 out of 119) of the studies were analyzable while the remainder of the studies had poor brachial artery image quality. In summary, there were no significant changes in the resting BAD, post-cuff BAD and %FMD associated with the exposures and data were inconclusive.

There are several limitations in interpretation of our data. First, these data represent a small and homogenous population of subjects which in turn, provides effect estimates with wide confidence intervals. Second, all three methods of measurement of endothelial function exhibit a relatively high variability as they represent either physiological or biochemical methods, making the assessment of effect of exposure difficult. Third, the results may not be generalizable to the general population as the study group included young and healthy college students only. Fourth, the outcome data are limited to effects measured at a single time point i.e 2 hours following exposure. Fifth, although subjects were blinded to the identity of the exposures, DE and SOA both have mild characteristic odors at the concentrations generated, and thus could have been distinguished from CA, but not readily from one another. However, previous experience

with these exposures has not suggested a robust symptomatic response that might engender a generalized physiologic response. [26, 27] Sixth, we could not control for confounding effects due to differences in diet, physical activity and other sources of exposure prior to the testing. Despite these limitations, the study had a methodological strength i.e. the use of a double-blind, randomized cross-over protocol to compare the effects of controlled exposures to two separate aerosols and a CA control. Since the response to exposure to DE/SOA particles was compared to the response to clean air within the same subject, the design offered the possibility of a more precise assessment of the exposure effect due to minimization of subject-specific variations.

In conclusion, our study demonstrates the utility of plasma nitrite as a reasonable marker for studying the changes in endothelial function in response to air pollutants. Future studies should investigate the changes in this marker in larger controlled exposure studies as well as changes following a longer time interval such as 6 or 12 hours post exposure. In addition, the study also concluded that the EndoPAT device may not be a useful method to capture the acute changes in endothelial function in response to air pollutants within a short time frame.

Tables

Table 1. Time Points for Outcome Assessment

Visit Number	Exposure Condition	Pre-exposure or Baseline	Immediate Post-exposure
1	DE	X	X
2	SOA	X	X
3	CA	X	X

All outcomes were assessed six times for each subject as depicted by a letter 'X' in the table.

Table 2. Summary of Available Data on Each Outcome

Outcome	Outcome Variables	Exposure Comparison	No. of Subjects	No. of Observations
EndoPAT	PAT Ratio	DE with CA	45	180
	Baseline PWA	SOA with CA	33	132
Plasma Nitrite	Plasma Nitrite Level	DE with CA	42	168
		SOA with CA	28	112
Brachial Artery Ultrasound Scanning	Resting BAD Post-cuff deflation BAD BAFMD	DE with CA	14	56

PWA, pulse wave amplitude
BAD, Brachial artery diameter
BAFMD, Brachial artery flow mediated dilation

Table 3. Mean, Minimum, and Maximum Pollutant Concentrations for Each Exposure Session During the Study

	Statistical Results	Mass (ug/m3)	Particle number (#/cm3)	Total hydrocarboons (ppm)		O_3 (ppm)		CO (ppm)		NO (ppm)		NOx (ppm)	
		Avg	Avg	Bkgrd	Avg	Bkgrd	Avg	Bkgrd	Avg	Bkgrd	Avg	Bkgrd	Avg
CA	N	49	49	43	43	43	43	48	48	49	49	49	49
	Mean	4.8	3460	0.54	0.71	0.02	0.02	0.93	0.93	0.07	0.06	0.20	0.19
	sd	3.9	1963	0.15	0.21	0.00	0.00	0.37	0.36	0.32	0.22	1.27	1.18
	Max	21.3	8549	0.87	1.17	0.02	0.03	1.99	1.96	2.04	1.45	8.93	8.32
	Min	1.0	842	0.07	0.14	0.01	0.00	0.44	0.50	0.00	0.00	0.00	0.00
DE	N	46	46	N/A	N/A	N/A	N/A	45	45	44	44	44	44
	Mean	192.7	72717	N/A	N/A	N/A	N/A	0.96	4.49	0.02	3.76	0.02	3.89
	sd	7.2	36053	N/A	N/A	N/A	N/A	0.40	1.24	0.02	1.20	0.02	1.14
	Max	206.3	137524	N/A	N/A	N/A	N/A	2.25	6.89	0.08	6.67	0.10	6.80
	Min	168.7	25976	N/A	N/A	N/A	N/A	0.50	1.59	0.00	1.49	0.01	1.67
SOA	N	43	43	43	43	43	43	N/A	N/A	N/A	N/A	N/A	N/A
	Mean	193.8	16076	0.56	0.84	0.02	0.02	N/A	N/A	N/A	N/A	N/A	N/A
	sd	10.7	5233	0.37	0.34	0.01	0.00	N/A	N/A	N/A	N/A	N/A	N/A
	Max	211.100	26431	2.47	2.28	0.02	0.03	N/A	N/A	N/A	N/A	N/A	N/A
	Min	148.600	6865	0.04	0.36	0.00	0.01	N/A	N/A	N/A	N/A	N/A	N/A

Table 4. Demographic and Clinical Characteristics of Study Subjects

Characteristic	EndoPAT Subset	Nitrite Subset	BAFMD Subset
N	45	42	14
Age (Years)	21.4 ± 3.2	21 ± 2.9	20.5 ± 2
Gender (M:F)	36:9 (80%)	33:9 (78.6%)	13:1 (80%)
Race (%)*			
White	36 (81.8%)	33 (78.6%)	14 (100%)
Black	7 (15.9%)	8 (19.1%)	0 (0%)
Asian	1 (2.3%)	1 (2.4%)	0 (0%)
Systolic BP* (mmHg)	116.5 ± 12.2	116.4 ± 12.3	120.5 ± 13.5
Diastolic BP* (mmHg)	70.3 ± 9.4	69.3 ± 9	70.4 ± 6.8
Weight (in pounds)*	166.6 ± 36.4	162.5 ± 36.9	189.4 ± 40.2
Body Mass Index* (kg/m^2)	25.2 ± 4.7	24.7 ± 4.7	27.7 ± 5.7
Serum Cholesterol (mg/dL)	157.6 ± 32.6	154.1 ± 30.7	151.6 ± 22.1
HDL Cholesterol (mg/dL)	51.8 ± 13.5	51.8 ± 13.6	49.3 ± 15.6
LDL Cholesterol (mg/dL)	83.7 ± 29.7	81.3 ± 26.1	76.9 ± 19.5
Serum Triglyceride (mg/dL)	110.2 ± 67	104.6 ± 56.6	126.4 ± 63.2

*Missing data

Values represent mean (± SD) unless otherwise specified.

Table 5. Changes in Mean PAT Measurements Before and After Exposure to Air Pollutants and Clean Air

Time Point	N	PAT Ratio	$PWA_{control}$	$PWA_{Occlusion}$
DE Day				
Pre-DE PAT test	45	1.66 ± 0.4	579.5 ± 455.9	561.6 ± 427.5
Post-DE PAT test	45	1.87 ± 0.5	199.1 ± 167.2	195.7 ± 166.9
$Diff_{DE}$ (Post – Pre)	45	0.20 ± 0.6	-380.4 ± 358.5	-365.8 ± 347.9
p-value*		0.02	< 0.0001	< 0.0001
CA Day				
Pre CA PAT test	45	1.65 ± 0.4	623.1 ± 435.5	622.9 ± 406.0
Post CA PAT test	45	1.88 ± 0.5	190.7 ± 164.4	199.1 ± 161.0
$Diff_{CA}$(Post – Pre)	45	0.23 ± 0.5	-432.4 ± 380.6	-423.8 ± 374.1
p-value *		0.004	< 0.0001	< 0.0001
SOA Day				
Pre-SOA PAT test	33	1.68 ± 0.5	517.1 ± 406.5	552.8 ± 375.3
Post-SOA PAT test	33	1.87 ± 0.5	206.2 ± 174.6	216.2 ± 202.9
$Diff_{SOA}$ (Post – Pre)	33	0.19 ± 0.6	-310.9 ± 290.8	-336.6 ± 281.7
p-value *		0.08	< 0.0001	< 0.0001
CA Day				
Pre CA PAT test	33	1.73 ± 0.5	621.5 ± 428.0	640.0 ± 401.5
Post CA PAT test	33	1.98 ± 0.5	186.4 ± 161.6	184.7 ± 137.8
$Diff_{CA}$(Post – Pre)	33	0.25 ± 0.5	-435.2 ± 374.1	-455.4 ± 368.0
p-value *		0.009	< 0.0001	< 0.0001

* For comparisons of PAT indices within each day using a paired t-test
Values represent mean ± SD.

Table 6. Pre to Post-Exposure Change in the PAT Measurements Associated with the Exposure to Air Pollutants

Outcome Variable	N	Change in PAT Measurements (Post-Pre Exposure)	95%CI	p-Value
PAT Ratio				
DE vs. CA	45	-0.02	(-0.23, 0.19)	0.86
SOA vs. CA	33	-0.06	(-0.33, 0.20)	0.63
DE or SOA vs. CA*	31	-0.01	(-0.24, 0.22)	0.91
PWA of Occlusion arm				
DE vs. CA	45	56.0	(-46.9, 158.9)	0.28
SOA vs. CA	33	118.8	(23.3, 214.2)	0.02
DE or SOA vs. CA*	31	62.0	(-34.6, 158.6)	0.20
PWA of Control arm				
DE vs. CA	45	50.2	(-43.0, 143.4)	0.28
SOA vs. CA	33	124.3	(56.6, 191.9)	0.0007
DE or SOA vs. CA*	31	64.3	(-19.1, 147.7)	0.13

* Complete data on all three exposure conditions were available in 31 subjects only.
CI, Confidence Interval
Models were adjusted for the order of visit.

Table 7. Post-Exposure Change in PAT Measurements Associated with the Exposure to Air Pollutants

Outcome Variable	N	Post-Exposure PAT Measurements	95%CI	p-value
PAT Ratio				
DE vs. CA	45	-0.01	(-0.16, 0.13)	0.86
SOA vs. CA	33	-0.10	(-0.28, 0.07)	0.25
DE or SOA vs. CA*	31	-0.05	(-0.19, 0.09)	0.50
PWA of Occlusion arm				
DE vs. CA	45	8.49	(-37.4, 54.4)	0.71
SOA vs. CA	33	52.2	(-11.0, 115.3)	0.10
DE or SOA vs. CA*	31	35.9	(-17.6, 89.4)	0.18
PWA of Control arm				
DE vs. CA	45	18.1	(-26.2, 62.5)	0.41
SOA vs. CA	33	47.0	(3.3, 90.6)	0.04
DE or SOA vs. CA*	31	37.8	(-3.5, 79.2)	0.07

* Complete data on all three exposure conditions were available in 31 subjects only.
CI, Confidence Interval
Models were adjusted for the pre-exposure measurement and the order of visit.

Table 8. Changes in PAT Measurements Associated with the Exposure to Air Pollutants After Eliminating the Effect of the Order of Visit

Outcome Variable	N	Post-Pre Exposure PAT Measurements	95%CI	p-Value
PAT Ratio				
DE vs. CA	10	-0.01	(-0.50, 0.48)	0.96
SOA vs. CA	11	-0.27	(-0.84, 0.30)	0.32
PWA of Occlusion arm				
DE vs. CA	10	143.8	(5.86, 281.8)	0.04
SOA vs. CA	11	63.5	(-93.5, 220.6)	0.39
PWA of Control arm				
DE vs. CA	10	122.0	(-7.4, 251.4)	0.06
SOA vs. CA	11	37.0	(-88.1, 162.0)	0.53
Outcome Variable	N	Post-Exposure PAT Measurements	95%CI	p-Value
PAT Ratio				
DE vs. CA	10	-0.04	(-0.44, 0.35)	0.80
SOA vs. CA	11	-0.20	(-0.60, 0.20)	0.28
PWA of Occlusion arm				
DE vs. CA	10	15.9	(-38.5, 70.4)	0.52
SOA vs. CA	11	59.8	(-68.9, 188.6)	0.32
PWA of Control arm				
DE vs. CA	10	49.6	(14.1, 85.2)	0.01
SOA vs. CA	11	6.00	(-105.2, 117.2)	0.91

Table 9. Changes in Mean Resting Brachial Artery Diameter Before and After the Exposure to Air Pollutants

Time Point	Resting BAD, Original data N=14	Resting BAD, Subset 1 N=12	Resting BAD, Subset 2 N=8
DE Day			
Pre-DE BAD	3.77 ± 0.5	3.68 ± 0.4	3.79 ± 0.4
Post-DE BAD	3.73 ± 0.4	3.64 ± 0.4	3.72 ± 0.3
$Diff_{DE}$ (Post – Pre)	-0.03 ± 0.16	-0.04 ± 0.17	-0.07 ± 0.19
p-value*	0.42	0.44	0.31
CA Day			
Pre CA BAD	3.83 ± 0.5	3.74 ± 0.5	3.82 ± 0.5
Post CA BAD	3.73 ± 0.4	3.71 ± 0.4	3.78 ± 0.4
$Diff_{CA}$(Post – Pre)	-0.10 ± 0.19	-0.04 ± 0.12	-0.03 ± 0.14
p-value*	0.08	0.33	0.54

* For comparisons of BAD within each day (pre and post) using a paired t-test
Values represent mean ± SD.

Table 10. Changes in Resting BAD Associated with the Exposure to Air Pollutants

Resting BAD	N	Post-Pre Exposure †	95%CI	p-Value
Original data	14	0.03	(-0.12, 0.17)	0.71
Subset 1	12	-0.02	(-0.15, 0.11)	0.75
Subset 2	8	-0.07	(-0.27, 0.13)	0.40
Resting BAD	N	Post-Exposure‡	95%CI	p-Value
Original data	14	0.02	(-0.11, 0.15)	0.74
Subset 1	12	-0.02	(-0.14, 0.10)	0.65
Subset 2	8	-0.07	(-0.25, 0.12)	0.39

CI, Confidence Interval

† Models were adjusted for the order of visit.

‡ Models were adjusted for the pre-exposure measurement and the order of visit.

Table 11. Changes in Mean Post-Cuff Deflation BAD Before and After the Exposure to Air Pollutants

Time Point	Post-cuff deflation BAD, Original data N=14	Post-cuff deflation BAD, Subset 1 N=12	Post-cuff deflation BAD, Subset 2 N=8
DE Day			
Pre-DE	3.96 ± 0.5	3.89 ± 0.4	4.00 ± 0.4
Post-DE	4.00 ± 0.5	3.90 ± 0.4	3.97 ± 0.3
$Diff_{DE}$ (Post – Pre)	0.04 ± 0.2	0.007 ± 0.21	-0.03 ± 0.22
p-value*	0.51	0.92	0.69
CA Day			
Pre CA	4.1 ± 0.5	4.02 ± 0.5	4.07 ± 0.4
Post CA	4.0 ± 0.5	3.98 ± 0.4	4.08 ± 0.5
$Diff_{CA}$(Post – Pre)	-0.08 ± 0.3	-0.04 ± 0.3	0.016 ± 0.23
p-value*	0.29	0.63	0.85

* For comparisons of post-cuff deflation BAD within each day (pre and post) using a paired t-test
Values represent mean ± SD.

Table 12. Change in Post-Cuff Deflation BAD Associated with the Exposure to Air Pollutants

Post-cuff deflation BAD	N	Post – Pre Exposure[†]	95%CI	p-value
Original data	14	0.06	(-0.13, 0.25)	0.52
Subset 1	12	0.02	(-0.19, 0.22)	0.87
Subset 2	8	-0.08	(-0.36, 0.20)	0.50
Post-cuff deflation BAD	N	Post-Exposure[‡]	95%CI	p-value
Original data	14	0.05	(-0.14, 0.25)	0.55
Subset 1	12	0.01	(-0.21, 0.23)	0.92
Subset 2	8	-0.08	(-0.39, 0.22)	0.52

CI, Confidence Interval

[†] Models were adjusted for the order of visit.

[‡] Models were adjusted for the pre-exposure measurement and the order of visit.

Table 13. Changes in FMD% Before and After the Exposure to Air Pollutants

Time Point	FMD%, Original data N=14	FMD%, Subset 1 N=12	FMD%, Subset 2 N=8
DE Day			
Pre-DE %FMD	5.3 ± 2.2	5.8 ± 1.9	5.6 ± 2.1
Post-DE %FMD	7.2 ± 2.7	7.0 ± 2.7	6.8 ± 3.0
$Diff_{DE}$ (Post – Pre)	1.9 ± 3.1	1.2 ± 2.8	1.2 ± 2.7
p-value*	0.04	0.16	0.26
CA Day			
Pre CA %FMD	6.8 ± 3.6	7.4 ± 3.5	6.7 ± 3.5
Post CA %FMD	7.1 ± 3.6	7.2 ± 3.8	7.7 ± 3.9
$Diff_{CA}$(Post – Pre)	0.3 ± 5.9	-0.3 ± 6.2	1.0 ± 6.3
p-value*	0.85	0.89	0.68

* For comparisons of FMD within each day (pre and post) using a paired t-test

Table 14. Change in FMD% Associated with Exposure to DE and CA Aerosols

FMD	N	Post- pre-Exposure †	95%CI	p-value
Original data	14	0.97	(-2.65, 4.58)	0.57
Subset 1	12	1.14	(-3.04, 5.32)	0.56
Subset 2	8	0.18	(-5.56, 5.93)	0.94
FMD	N	Post-Exposure ‡	95%CI	p-value
Original data	14	-0.79	(-3.52, 1.93)	0.53
Subset 1	12	-1.00	(-4.03, 2.02)	0.47
Subset 2	8	-2.01	(-6.79, 2.77)	0.33

CI, Confidence Interval

† Models were adjusted for the order of visit.

‡ Models were adjusted for the pre-exposure measurement and the order of visit.

Table 15. Changes in Mean Plasma nitrite concentration Before and After Exposure to Air Pollutants and Clean Air

Time Point	N	Plasma Nitrite, Mean ± SD
DE Day		
Pre-DE	42	131.3 ± 69.9
Post-DE	42	127.3 ± 58.4
Diff (Post – Pre)	42	-4.03 ± 57.6
p-value*		0.65
CA Day		
Pre-CA	42	129.8 ± 69.2
Post-CA	42	132.7 ± 78.9
Diff (Post – Pre)	42	3.0 ± 49.6
p-value*		0.70
SOA Day		
Pre-SOA	28	135.1 ± 43.1
Post-SOA	28	123.8 ± 43.7
Diff (Post – Pre)	28	-11.4 ± 38.6
p-value*		0.13
CA Day		
Pre-CA	28	146.2 ± 77.7
Post-CA	28	148.7 ± 90.6
Diff (Post – Pre)	28	2.6 ± 58.4
p-value*		0.82

* For comparisons of plasma nitrite within each day (pre and post) using a paired t-test

Table 16. Change in Plasma nitrite concentration Associated with the Exposure to Air Pollutants

Post minus pre exposure plasma nitrite	N	Post –Pre Exposure †	95%CI	p-value
DE vs. CA	42	-6.9	(-26.4, 12.6)	0.48
SOA vs. CA	28	-13.8	(-40.2, 12.7)	0.29
DE or SOA vs. CA*	28	-10.7	(-33.0, 11.5)	0.34
Post-exposure plasma nitrite	N	Post-Exposure‡	95%CI	p-value
DE vs. CA	42	-5.6	(-23.2, 12.0)	0.52
SOA vs. CA	28	-16.4	(-41.6, 8.9)	0.19
DE or SOA vs. CA*	28	-12.3	(-32.8, 8.2)	0.23

CI, Confidence Interval

* Complete data on all three exposure conditions were available in 28 subjects only.

† Models were adjusted for the order of visit.

‡ Models were adjusted for the pre-exposure measurement and the order of visit.

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CHANGES IN ENDOTHELIAL FUNCTION MARKERS ASSOCIATED WITH ACUTE INCREASES IN AMBIENT AIR POLLUTANT CONCENTRATIONS

by

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ABSTRACT OF MANUSCRIPT 3 OF 3

CHANGES IN ENDOTHELIAL FUNCTION MARKERS ASSOCIATED WITH ACUTE INCREASES IN AMBIENT AIR POLLUTANT CONCENTRATIONS

MARKERS

Dissertation Director:

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ABSTRACT

Introduction: The role of endothelial dysfunction has been suggested as a potential mechanism between an exposure to ambient air pollutants and adverse cardiovascular outcomes. We examined this association using two novel markers of endothelial function in a panel study.

Methods: We linked hourly concentrations of five criteria air pollutants [particle mass < 2.5 μm ($PM_{2.5}$) in aerodynamic diameter, carbon monoxide (CO), sulphur dioxide (SO_2), nitrogen dioxide (NO_2) and ozone (O_3)] measured at a monitoring site closest to the Busch Campus of the Rutgers University in the state of New Jersey to plasma nitrite concentration and EndoPAT measurements obtained from 52 young, healthy and non-smoking college students for up to 7 preceding days. The changes in plasma nitrite concentration, pulse wave amplitude (PWA) and PAT ratio associated with an interquartile (IQR) range increase in pollutant concentration were examined using linear mixed models.

Results: Each IQR increase in the mean $PM_{2.5}$ and CO concentration in the first 24 hours before the endothelial function measurement was associated with a significant increase in

plasma nitrite concentration by 17.1% (15.5 nM; 95% CI: 2.4, 28.5 nM) and 17.2% (15.6 nM; 95% CI: 2.4, 28.9 nM), respectively. We also observed a delayed significant increase in plasma nitrite concentration associated with each IQR increase in O_3 and SO_2 concentration over lag day 4 and 5 by 15.8 % (14.4 nM; 95% CI:1.0, 27.7 nM) and 15.7% (14.3 nM; 95% CI: 3.4, 25.3 NM), respectively. The PAT ratio or PWA did not exhibit substantial changes associated with each IQR increase in any of the pollutant concentrations.

Conclusions: These data suggest that an exposure to ambient air pollutants stimulates an inflammatory mechanism via inducible nitric oxide synthase and generates nitric oxide within a short period of time. To our knowledge, this is the first study to explore the effects of ambient air pollutants on endothelial function using plasma nitrite concentration as an indicator.

CHANGES IN ENDOTHELIAL FUNCTION MARKERS ASSOCIATED WITH ACUTE INCREASES IN AMBIENT AIR POLLUTANT CONCENTRATIONS

Introduction

A growing body of literature has confirmed an acute and consistent association between an increased ambient air pollutant concentration and increased hospital admissions as well as deaths due to cardiovascular (CV) diseases.[1-13] Elevated levels of pollutants such as ambient particulate matter < 2.5 μm in aerodynamic diameter ($PM_{2.5}$), ozone, sulphur dioxide, nitrogen dioxide and elemental carbon have been shown to be acutely associated with adverse CV events.[14-18] Specifically, investigators have observed significantly increased risk of ventricular arrhythmia[14, 15], paroxysmal atrial fibrillation (PAF),[16] and myocardial infarction (MI) [17, 18] associated with increase in the above-mentioned criteria air pollutants in 24 hours before the CV event.

There is also strong evidence that the adverse cardiovascular events associated with elevated levels of air pollutants can be observed rapidly, i.e. within a few hours prior to the CV event. [16, 17, 19, 20, 21] Peters et al (2001) showed that the risk of MI associated with an increase of 25 mg/m^3 $PM_{2.5}$ was elevated by 48% during a 2-hour period before the onset of MI.[17] Using a case-crossover study design, Rich et al (2006) reported a statistically significant two-fold increase in the risk of PAF episodes associated with each 22-parts per billion increase in the mean ambient ozone concentration in the hour before the arrhythmia[16], thereby suggesting that air pollution may precipitate such events in a comparatively short period of time. Furthermore, it has also been shown that

cumulative exposures over a few days prior to any vascular event may be a better predictor of such events.[22] Therefore, some researchers took a step further and explored the effects of air pollutants up to the previous 6 days in addition to the same day association.[23, 24]

Although several mechanisms may operate concurrently in response to inhalation of pollutants, many epidemiological, clinical and toxicological studies have suggested a role of altered endothelial function in causing acute and chronic cardiovascular outcomes.[23, 25-27] These studies utilized changes in brachial artery flow-mediated dilation (BAFMD) as an endpoint measure of endothelial function. For instance, Dales et al reported a 0.48% reduction in percent BAFMD associated with increases in 2-hour mean $PM_{2.5}$ concentration among 39 healthy volunteers.[25] A panel study of 270 subjects with diabetes or at risk for diabetes examined the changes in BAFMD associated with ambient pollutant concentrations, specifically of $PM_{2.5}$, sulphates, black carbon and particle number count, on the same and previous few days. [23] They found that an IQR increase in six day moving averages of black carbon and sulphate concentrations were associated with a 12.6% and 10.7% decrease in BAFMD. Additionally, Rundell et al reported brachial artery vasoconstriction and a decrease in BAFMD after subjects inhaled a high dose of PM while exercising (143,501 ± 58,565 particles cm−3)..[26]These studies suggested that acute exposures to ambient air pollutants may produce endothelial dysfunction, or vascular smooth muscle dysfunction and either reduce endothelial production of nitric oxide (NO) or cause depletion of NO due to production of oxidative radicals resulting from particle-associated inflammation and oxidative stress.[23] Contrary to these reports, Liu et al showed that percent BAFMD

increased following subjects' personal exposure to PM_{10} for 24 hours, which they attributed to increased parasympathetic activity.[27]

In order to further explore the mechanistic link of endothelial dysfunction, we sought to examine the changes in two novel markers of endothelial function, namely plasma nitrite concentration and EndoPAT measurements associated with increases in five criteria air pollutant concentrations on the preceding 7 days using a panel study design. The specific aims of our study were to (1) examine the changes in plasma nitrite concentration associated with increases in five criteria air pollutant concentrations, namely $PM_{2.5}$, carbon monoxide (CO), sulphur dioxide (SO_2), nitrogen dioxide (NO_2) and ozone (O_3) on the preceding 7 days, (2) examine the changes in EndoPAT measurements, namely pulse wave amplitude (PWA) and PAT ratio, associated with increases in $PM_{2.5}$, CO, SO_2, NO_2, and O_3 over the preceding 7 days.

Materials and Methods

Study Population

This study is a secondary data analysis using data from a double-blind crossover study conducted at Environmental and Occupational Health Sciences Institute (EOHSI) in Piscataway, New Jersey. The crossover experiment was conducted in order to explore two mechanistic pathways, namely endothelial dysfunction and activation of platelets as operating mechanisms by which a short-term exposure to air pollutants causes adverse cardiovascular effects. This experiment examined the changes in the markers of platelet activation and endothelial function associated with 2-hour exposures to two kinds of air pollution particles, namely diesel exhaust (DE) and secondary organic aerosol (SOA),

relative to filtered clean air (CA) in a group of 63 healthy and non-smoking young college students (ages of 18-30 years). Data on both outcomes were collected prior to and following the 2-hour exposures. The baseline measures obtained prior to the exposure to air pollutants are the focus of the current secondary data analysis. The study was approved by the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School Institutional Review Board. The subjects were recruited from the Busch campus of the Rutgers University community using flyers during the years 2005-2009. A detailed description of the recruitment process, exclusion/inclusion criteria and clinical evaluation of the subjects has been outlined in manuscript two.

Study Protocol

Each subject underwent measurement of study outcomes one day per week over 3 consecutive weeks. Subjects were assigned to receive each of the three exposure conditions, i.e. DE, SOA and CA in a random order where sessions were conducted at least one week apart. All outcomes including assessment of endothelial function were evaluated at baseline, i.e. within 1–hour before entering the controlled environmental facility (CEF) and immediately following a 2-hour exposure to DE, SOA and CA. On the day of testing, subjects reported to the Clinical Center at 8:00 A.M. Subjects were instructed to arrive following an overnight fast beginning at 12:00 midnight the day before testing. They were also instructed to abstain from: (1) any caffeinated and/or alcoholic beverages and vigorous exercise 12 hours prior to the testing, and (2) aspirin containing medications 2 weeks prior to the testing. A staff member performed a check-in to ascertain that the above conditions had been met. A pre-exposure blood sample was collected into vacuum tubes and aliquotted for measurement of plasma nitrite and other

biomarkers such as markers of platelet activation, platelet aggregation, and activity of ubiquitin/proteasome pathway. Then endothelial function was measured using two methods simultaneously, namely, the EndoPAT tonometer and brachial artery ultrasound scanning (See Appendix for methods of data collection), approximately between 8:30 A.M and 9:00 A.M. Subjects were then taken to the CEF and were exposed for 2 hours to either DE (200 $\mu g/m^3$), SOA (200 $\mu g/m^3$), or CA in a random order. Immediately following the 2-hour exposure session, all measurements were repeated.

Outcome Measurements

Effects of air pollutants were assessed on three outcome measurements, namely plasma nitrite concentration, pulse wave amplitude (PWA) for the occlusion arm and PAT ratio where the latter two were obtained from the EndoPAT device. A detailed description of the methods of data collection and study variables obtained from the EndoPAT device is provided in the appendix. The EndoPAT device measured PWA for the control arm separately. However, since the tone of peripheral blood vessels as measured by PWA was not found to vary between the control and occlusion arms, we decided to use PWA obtained from the occlusion arm alone. This was done to avoid redundancy of study variables measuring the same endpoint. These data were collected from 52 subjects at baseline prior to the exposure to air pollutants in the CEF. Although the primary crossover experiment enrolled 63 subjects, data using the EndoPAT device were available on only 52 (82.5% of the total study sample) subjects. This was because the EndoPAT test was added to the experimental design 8 months after the initiation of the primary experiment. The study period during which the exposure sessions were

conducted, extended from August 1, 2006 till May 31, 2009. The dataset comprised of 139 observations where only 37 out of the 52 subjects completed all 3 visits while the rest of the 15 subjects completed either 1 or 2 visits.

Air Pollution

Ambient concentrations of five gaseous criteria air pollutants, namely $PM_{2.5}$, CO, SO_2, NO_2, and O_3 measured hourly at up to 14 monitoring sites throughout the state of New Jersey were retrieved from the U.S. Environmental Protection Agency (EPA) Web site[28] for the entire study period. The pollutants are generated primarily from local and regional combustion sources (power plants, automobiles, and trucks). The total number, location and geographic range of monitors changed minimally during the years of the study though some monitors were discontinued, and others were added. For $PM_{2.5}$, there were at least 8 monitoring sites in NJ.[29] Similarly, there were 14, 13, 11 and 8 monitoring stations for O_3, SO_2, CO, NO_2, respectively. Since the study subjects either lived on the Busch Campus of the Rutgers University in Piscataway or in close proximity to the campus at the time of their study participation, a monitor closest to the campus was selected to represent subjects' exposure to ambient air pollutants during the study. This, however, was a limitation of the study design as we did not collect information on subjects' exact location in the few days prior to their study participation and assumed that they spent most of their time on the campus at the time of their study participation. Table 1 provides a description of the location of the monitors and their distance from EOHSI where the study was conducted. For each day during the study period, we assigned pollutant concentrations beginning at 8 A.M. on that day, since the outcome

measurements were obtained between 8 - 9 A.M. as described earlier. These data were then linked to the outcome data by date and hour of the day. Thus, we obtained pollutant concentrations for 168 hours prior to 8 A.M. on the day of outcome measurement. We calculated 7 moving averages of pollutant concentrations as follows: mean of hours 0-23 (lag day 1), mean of hours 24-47 (lag day 2), mean of hours 48-71 (lag day 3), mean of hours 72-95 (lag day 4), mean of hours 96-119 (lag day 5), mean of hours 120-143 (lag day 6) and mean of hours 144-167 (lag day 7). If greater than 25% of the total hours of pollutant concentrations were missing, we set the mean for that lag period to missing. We used these mean concentrations in main statistical analyses.

Potential Confounders

The variables that may be correlated with ambient air pollutant concentrations and that are independent predictors of outcomes under study are possible confounders. Some examples of confounders are meteorological measurements such as ambient temperature and relative humidity levels during the same times as the lagged pollutant concentrations, calendar month, day of the week and year. The factors such as temperature and relative humidity are associated with ambient air pollutant concentrations. In New Jersey, hourly temperature and relative humidity measurements were made at the Newark, Caldwell, Somerset, and Trenton airports during the study period.[30] We used the measurements from a monitor located at the Newark International Airport approximately 27 miles from the EOHSI during the study period. We calculated lagged mean temperature and relative humidity levels in the same manner as the pollutant concentrations and used these values in all analyses.

Several studies have previously described variations in ambient pollutant concentrations by calendar month or season.[31-34] A pattern of high levels in winter season (December-February) and low levels in summer months (May-August) has been reported in NO_2, $PM_{2.5,}$ and CO concentrations. There is considerably less mixing of pollutants in the winter months, which ultimately lead to elevated levels. Several meteorological factors such as wind speed, height of the inversion as well as changes in traffic patterns by season are considered to contribute to the seasonal variations. Furthermore, diurnal variations as well as variations in pollutant concentrations by the day of the week (weekday versus weekend) have been discussed. [34, 35] Peak CO concentrations have been reported during morning and evening traffic rush hours. Similarly, pollutant concentrations are reported to be low during weekend compared to weekdays.[34, 35] Moreover, endothelial function as measured by BAFMD has been known to display seasonal variations as well as variations due to temperature and humidity.[36, 37] Thus, the statistical analyses as described below were needed to account for their possibly confounding effects.

Statistical Analyses

We summarized the demographic and clinical characteristics of the study subjects using means and standard deviations or percentages, as appropriate. Next, we calculated means, standard deviations and interquartile ranges (IQR) to examine the distribution of pollutant concentrations and weather parameters for the entire study period. Next, linear mixed models were fit to examine the change in outcomes [and their 95% confidence intervals (CI)]associated with an IQR increase in mean temperature and relative humidity levels

measured during the same times as lagged pollutant concentrations. Linear mixed models were also fit to examine the change in outcomes associated with calendar month, year and day of the week. Although only mean temperature in the first 24 hours prior to the outcome measurement, calendar month and year produced the largest changes in outcomes, we fit linear mixed models including all confounding variables (i.e. temperature, relative humidity levels, calendar month, year and day of the week) to assess the best fit of the model explaining the relationship between pollutant concentration and outcome. We conducted likelihood ratio tests and selected the best fit of the model based on the criteria of minimizing Akaike's Information Criterion. Thus, the final models adjusted only for the effects of mean temperature over 0-23 hours, as well as calendar month and year. We assessed whether a linear trend was adequate to describe the relationship between temperature and outcomes. This was done by examining scatterplots plotted for each lag of temperature with each of the outcomes separately. We observed a weak negative linear association between temperature and plasma nitrite. A weak positive linear association was observed between temperature and PWA/PAT ratio. After confirming the linear nature of this relationship, linear mixed models were fit to examine the change in plasma nitrite, PWA and PAT ratio associated with increases in the pollutant concentrations over 7 lagged hour intervals (0-23, 24-47, 48-71, 72-95, 96-119, 120-143 and 144-167 hours) after adjusting for three confounders, i.e. mean temperature in the first 24 hours, calendar month and year. A random effect for each subject induced a compound symmetry between observations taken on the same subject. The changes in outcomes and their 95% CI's obtained from the models were scaled to the IQR of the 24-hour/daily mean pollutant concentrations observed during the study period. In addition to

the main analyses, we performed four sensitivity analyses. First, we adjusted for lagged temperature using the same lags as the pollutant, for example, we adjusted for mean temperature over lag day 2 (24-47 hours) in the model examining the change in outcomes associated with pollutant concentration on lag day 2 (24-47 hours). This was done to examine if the changes in outcomes obtained using this method were different from the models adjusted for the effect of mean temperature over the first 24 hours (0-23 hours). Second, we redefined the pollutant lags using a cumulative method (i.e. mean of hours 0-23, mean of hours 0-47, mean of hours 0-71, mean of hours 0-95, mean of hours 0-119, mean of hours 0-143, and mean of hours 0-167). This was done to assess if the pollutant concentrations had any cumulative effects on the outcomes instead of examining the effect on individual lagged days. Third, we fit two pollutant models for those pollutants only where increased concentrations in specific lags were associated with an increase in plasma nitrite in the single pollutant model. This was done to evaluate whether each pollutant effect observed in the single pollutant model was independent of other pollutants. Finally, since the study included repeated measures within subject (each subject contributed to at least 1 and up to 3 outcome measurements) and since the average exposures were different across subjects due to their staggered entry into the study over a 3.5 years period, it may have been possible that the between and within-subject changes in the outcomes associated with pollutants were different in strength and direction (for example, as average exposure to $PM_{2.5}$ in the first 24 hours increases by 1 unit between two subjects, plasma nitrite increases by 10 units. On the other hand, as average exposure to $PM_{2.5}$ in the first 24 hours increases by 1 unit within two visits of the same subject, plasma nitrite decreases by 15 units).[38, 39] We assessed this possibility by

fitting two separate terms for between and within-subjects effects each in the models fit for those pollutants where increased concentrations were associated with an increase in plasma nitrite.[38] By doing so, we did not assume that the between and within-subjects effects of pollutants were equal. In addition to these analyses, we stratified subjects by body mass index (BMI) and examined whether an increase in plasma nitrite associated with increased concentrations of pollutants was more prominent in overweight/obese group of subjects compared to the subjects with a normal BMI. All analyses were conducted using the SAS programming language version 9.3 (SAS Institute, Inc, Cary, NC).

Results

Study Subjects

Table 2 provides baseline characteristics including demographic characteristics of the study subjects and their distribution. Approximately 80% of the subjects were White males. The mean age was 21.2 [±Standard Deviation =3.0] years. All clinical characteristics including systolic and diastolic blood pressures as well as lipid profile were within a normal clinical range except 32% of the subjects were overweight (BMI: 25-29.9) and 12% were obese (BMI ≥ 30).

Air Pollutant and Meteorological Measurements

The study period extended from August 1, 2006 toMay 31, 2009. The distributions of air pollutant concentrations and meteorological characteristics measured at the monitoring site closest to the Busch Campus of the Rutgers University in New Jersey during this

period, averaged hourly and daily, are summarized in Table 3. There were a total of 24,840 possible hours (or 1,035 days) during the study period. For all pollutants, ≤ 4% of the hourly concentrations were missing. Daily $PM_{2.5}$ concentrations had a median of 6.6 $\mu g/m^3$ (IQR =7 $\mu g/m^3$). Daily SO_2 and NO_2 concentrations exhibited a right skewed pattern with a median of 1.9 parts per billion (ppb) and an IQR of 2.2 ppb for SO_2 and a median of 11.5 ppb and an IQR of 9.6 ppb for NO_2. Daily O_3 and CO concentrations were normally distributed with a median of 24.8 ppb and an IQR of 17.1 ppb for O_3 and a median of 0.3 parts per million (ppm) and an IQR of 0.16 ppm for CO. Hourly mean pollutant concentrations exhibited similar distributional patterns (Table 3).

The distributions of pollutant concentrations as well as meteorological characteristics averaged daily, during the study period by season are summarized in Table 4. Mean daily $PM_{2.5}$, CO and O_3 concentrations were the highest during summer months while mean daily SO_2 and NO_2 concentrations were the highest during winter months. When the distribution was examined by season for hourly concentrations, similar patterns were observed (data not shown). As expected, the mean temperature was the highest during summer months and the lowest during winter months. Mean percent relative humidity was the highest during fall.

Correlations between Pollutants and Meteorological Measurements

Table 5 shows overall and by season Pearson correlation coefficients for individual pollutants and meteorological measurements. CO and $PM_{2.5}$ were moderately positively correlated and the correlations were positive for all seasons (overall r=0.45). SO_2 and $PM_{2.5}$ were moderately positively correlated and the correlation was stronger during

spring and winter months (overall r=0.42, in spring r=0.54, in winter r=0.64). NO_2 concentrations were positively correlated with CO and SO_2 and the correlation was stronger during spring and winter months (with CO: overall r=0.51, in spring and in winter r=0.61; with SO_2: overall r=0.53, in spring r=0.51 and in winter r=0.58). O_3 concentrations were positively correlated with temperature and the correlation was the strongest during summer months (overall r=0.51, in summer r=0.67). Moderate negative correlation existed between NO_2 and O_3 and the correlation was stronger in spring and winter months (overall r=-0.64, in spring r=-0.67, in winter r=-0.80). We also examined the correlations between pollutants in a subset of the entire study period when the outcomes were measured (N=139 days) and found them to be similar in magnitude and direction as those observed during the entire study period.

Pollutant Concentrations by hour of the day, day of the week, calendar month and year

We examined the mean pollutant concentrations by hour of the day, day of the week, calendar month and year. Figures 1 and 2 depict mean individual pollutant concentration by hour of the day. Mean NO_2 and CO concentrations exhibited two peaks representing traffic rush hours, one between 5 to 8 A.M. and the second between 4 to 8 P.M. Mean $PM_{2.5}$ and SO_2 concentration showed a peak between 5 to 8 A.M., but no appreciable peak was observed during the evening hours. Ozone concentrations were the highest during sunlight i.e. between 11 A.M. to 4 P.M. and the lowest between 4 to 6 A.M. Mean NO_2 concentrations followed an opposite pattern to ozone with the lowest concentrations observed during 11 A.M. to 4 P.M. Figures 3 and 4 depict mean pollutant concentrations

by day of the week. NO_2 and CO concentrations were the lowest whereas ozone concentrations were the highest on weekends (Saturday and Sunday) compared to weekdays (Monday to Friday). Mean $PM_{2.5}$ and SO_2 concentrations did not exhibit appreciable changes by day of the week. Figures 5 and 6 depict mean pollutant concentration by calendar month. Mean ozone concentrations were the highest during summer months (April to August) with a peak observed in the month of July and the lowest during winter months (January, November, December). Mean NO_2 concentrations exhibited an opposite trend to ozone with the highest concentrations during winter months. Mean $PM_{2.5}$ concentration was the highest during summer months (June-August). Mean SO_2 and CO concentrations exhibited no appreciable changes by calendar month. Figures 7 and 8 depict mean pollutant concentration by year. From year 2006 to 2009, mean O_3 concentrations showed an increase whereas mean CO concentrations showed a decrease. Other pollutants did not show much variation by year. In summary, we observed that pollutant concentrations vary by these factors and they should be considered as potential confounders in adjusted analyses.

Distribution of Outcome Measurements

The distributions of plasma nitrite concentration, PWA and PAT ratio are provided in Table 6. Of the 139 study sessions, plasma nitrite concentration was available for only 128 sessions. The mean (± Standard Deviation) plasma nitrite concentration was 130.8 (± 60.7) nanomolar (nM) and the distribution was right skewed. Both PWA and PAT ratio followed a right-skewed distribution. PAT ratios ranged from 1 to 3.3.

Association of Outcomes with Meteorological Measurements

We fit linear mixed models to estimate a change in the outcomes associated with each IQR increase in mean temperature and relative humidity levels. This was done to determine if mean temperature and relative humidity were significant predictors of the outcome, which in turn, would suggest their inclusion in adjusted analyses. The changes in outcomes associated with each IQR increase in temperature/relative humidity and their 95% confidence intervals (CI) are provided in Table 7. As the mean temperature increased by an IQR (i.e. 28.9 deg F) over lag day 1, lag day 2 and lag day 3, plasma nitrite concentration decreased significantly by 29.3, 27.3 and 22.9 nM respectively. PWA and PAT ratio increased significantly by 155.4 and 0.17 units with each IQR increase in the mean temperature over lag day 1, respectively. However, the changes observed in all outcomes associated with an IQR increase in relative humidity levels were smaller in magnitude compared to the changes associated with temperature and the estimates did not predict a specific trend toward a decrease or increase in the outcomes (Table 7).

Association of Outcomes with Other Potential Confounders

We fit linear mixed models to estimate a change in the outcomes associated with calendar month, year and day of the week. This was also done to determine if the above-mentioned covariates were significant predictors of the outcome and whether they needed to be included in adjusted analyses. The changes in outcomes associated with these covariates and their 95% CI's obtained from the models are provided in Table 8. PAT ratio was significantly greater in the months of March, May and September compared to

December [March versus Dec: 0.70, 95% CI: 0.33, 1.08; May versus December: 0.42, 95% CI: 0.06, 0.79, September versus Dec: 0.40, 95% CI: 0.03, 0.76]. Similarly, plasma nitrite concentration was significantly higher in year 2006 compared to year 2009 [78.0, 95% CI: 15.3, 140.7]. However, the changes in any of the outcomes did not differ substantially by day of the week.

Main Analyses

The changes in the outcomes associated with each IQR increase in the mean pollutant concentrations adjusted for mean temperature over 0-23 hours, calendar month and year and their 95% CI's are presented in Table 9 and figures 9-23. The absolute changes in outcomes and their 95% CI's are presented in Table 9 while the percent changes in outcomes and their 95% CIs are presented in figures 9-23. The percent changes were calculated with respect to the 25th percentile for the respective outcomes. For example, the absolute change in plasma nitrite associated with an IQR increase in $PM_{2.5}$ on lag day 1 was15.5 nM (95% CI: 2.4, 28.5). The percent change in plasma nitrite associated with an IQR increase in $PM_{2.5}$ on lag day 1 was 17.1% (95% CI: 2.6%, 31.4%) where the change was calculated with respect to its 25th percentile value of 90.9 nM. While both estimates represent the same change in plasma nitrite, a percent change as presented in the figures allows a reader to compare the changes between different outcomes or different pollutants. For example, a 17.1% increase in plasma nitrite is smaller in magnitude compared to a 17.6% increase in PWA associated with an IQR increase in $PM_{2.5}$ on lag day 1.

Plasma nitrite concentration increased significantly by 17.1% [15.5 nM (95% CI: 2.4, 28.5 nM)] and 17.2% [15.6 nM (95% CI: 2.4, 28.9 nM)] with each IQR increase in the mean $PM_{2.5}$ and the CO concentration over lag day 1 (0-23 hours), respectively (Figures 9 and 10). A significant but delayed increase by 15.8% [14.4 nM (95% CI: 1.0, 27.7 nM)] in plasma nitrite was also observed with each IQR increase in the mean O_3 concentration over lag day 4 (Figures 11). Similarly, an increase of 15.7% [14.3 nM (95% CI: 3.4, 25.3 nM)] in plasma nitrite was observed with each IQR increase in the mean SO_2 concentration over lag day 5 (Figures 12). No significant association between plasma nitrite and NO_2 concentration was observed (Figure 13). Also, we did not observe substantial changes in the PWA (Figures 14-18) or the PAT ratio (Figures 19-23) associated with an IQR increase in any of the pollutants. When subjects were stratified by BMI into two groups [normal BMI (< 25) and overweight/obese (BMI ≥ 25)], we found that plasma nitrite increased significantly among subjects with a normal BMI with each IQR increase in mean $PM_{2.5}$ over 0-23 hours [24.3 nM (95% CI: 4.6, 44.0 nM)] and O_3 concentration over 72-95 hours [22.9 nM (95% CI: 5.3, 40.4 nM)], but these effects disappeared in the overweight/obese subjects (Table 10).

Sensitivity Analyses

When we adjusted for lagged temperature using the same lags as the pollutant (for example, adjusting for mean temperature over lag day 2 in the model examining the change in outcomes associated with an IQR increase in the mean pollutant concentration on lag day 2), the changes in the outcomes were comparable to those produced in the main analyses (Table 11). For instance, plasma nitrite increased by 7.9 nM (95% CI: -3.7,

19.6) and by 7.3 nM (95% CI: -4.8, 19.3) with each IQR increase in mean $PM_{2.5}$ concentration over lag day 2 when the models were adjusted for mean temperature over lag day 1 (main analyses) and lag day 2 (sensitivity analyses), respectively.

We redefined the pollutant lags using a cumulative method to calculate the moving averages (i.e. mean of hours 0-23, mean of hours 0-47, till mean of hours 0-167) and recalculated the changes in outcomes associated with an IQR increase in the mean pollutant concentrations. The results obtained from this alternative method are presented in Table 12. Similar to the main analyses, a significant association was observed between plasma nitrite and $PM_{2.5}$, SO_2 and CO. Plasma nitrite concentration significantly increased with each IQR increase in mean $PM_{2.5}$ and CO over 0-23, 0-47, and 0-71 hours. Since we observed that the most significant effect of $PM_{2.5}$ occurred within the first 24 hours prior to the nitrite measurement in the main analyses, this effect was perhaps carried forward to the latter lags when the pollutant lags were redefined using the cumulative method and the effects seen in the 0-47 and 0-71 hours were primarily driven by the changes observed in the first 24 hours. The significant increase observed in plasma nitrite associated with an IQR increase in the mean O_3 concentration over lag day 4 disappeared when the pollutant lags were redefined.

Since we found a significant association between mean $PM_{2.5}$ and CO concentration over lag day 1 and plasma nitrite, we decided to explore if this stimulating effect was induced earlier than the first 24 hours using shorter time lags of 0-2 hours, 0-5 hours and 0-11 hours. Figures 24 and 25 present these two associations. We observed that the change in plasma nitrite associated with an IQR increase in the mean $PM_{2.5}$ and CO concentration over shorter time lags was in the same direction as the longer time lags, but

it failed to reach statistical significance. When we fit two pollutant models for those pollutants where increased concentrations in specific lags were associated with an increase in plasma nitrite in the single pollutant model, we found that the effect estimates obtained from the two pollutant models were as large as they were in the single pollutant models (Table 13). For instance, plasma nitrite increased significantly with each IQR increase in mean $PM_{2.5}$ concentration over 0-23 hours and this association remained significant even when mean SO_2 or mean O_3 concentration over 0-23 hours was added to the model.

Since the outcome measurements were obtained over an extended period of time, we examined if the between-subject effects of pollutants on plasma nitrite were different from within-subject effects (Table 14). We found that when the mean $PM_{2.5}$ concentration over the first 24 hours increased by an IQR, within two visits of the same subject, plasma nitrite increased by 19.6 nM (95% CI: 4.5, 34.7 nM). However, the increase in plasma nitrite associated with each IQR increase in the mean $PM_{2.5}$ concentration over the first 24 hours was much smaller in magnitude between two subjects. Greater within-subject increases were also observed in plasma nitrite associated with the mean CO and O_3 concentrations. These effects were attributable to differences within a subject than between subjects and further supported our hypothesis of acute effects of air pollutants.

Discussion

Our study demonstrated that with increased $PM_{2.5}$ and carbon monoxide concentrations in the first 24 hours before a blood sample for measurement of plasma nitrite concentration

was collected, plasma nitrite concentration increased significantly by approximately 17%, suggesting an acute effect of pollutants on endothelial function via an inflammatory pathway. In addition, we also observed a significant increase in plasma nitrite concentration associated with increase in the mean ozone and sulphur dioxide concentrations over lag day 4 and 5, respectively. Since nitrite (NO_2^-) is the major breakdown product of nitric oxide (NO) in plasma [40], the increase in plasma nitrite within a short period of time following the initial insult confirmed NO generation via several pathways. Since we did not observe substantial changes in the pulse wave amplitude or PAT ratio, the data further suggest that the increase in NO did not necessarily result in vasodilation (decreased vascular tone) and/or improvement of endothelial function.

NO is a soluble gas synthesized in various mammalian cells.[40, 41] Nitric oxide synthases (NOS) are the enzymes responsible for NO generation. Three distinct NOS isoforms have been identified to date: neuronal NOS (type I), inducible NOS (type II), and endothelial NOS (type III).[40] NO is the product of the five-electron oxidation of one of the chemically equivalent guanidinonitrogens of L-arginine by the NOS enzymes.[42] The three isoforms vary considerably in cellular location, structure, regulation, and functions. The endothelial NOS is mostly membrane bound and found only in endothelial cells. The neuronal NOS is identified in the cytosol of central and peripheral neurons.[43] Both isoforms are present in resting state of cells and upon stimulation, generate small amounts of NO instantaneously for short periods and the process is calcium dependent.[41] In contrast, the third isoform i.e. inducible NOS or iNOS, is not constitutively present, but instead can be induced via messenger RNA activation in circulating monocytes and airway epithelial cells, in the case of exposure to air pollutation.[44] Stimuli typically

include cytokines such as tumor necrosis factor-α, interleukins and interferon-γ as well as lipopolysaccharide, and after exposure to the stimulating agent (for example, exposure to pollutants, bacterial agents, etc), NO synthesis is delayed by several hours while enzyme and cofactors are synthesized. Contrary to the effects of endothelial and neuronal NOS, the NO synthesis by inducible NOS is sustained for several hours or longer.[41]

The role of systemic inflammation as one of the operating mechanisms has been suggested to explain the link between air pollution and adverse cardiovascular events such as arrhythmia, myocardial infarction, atherosclerosis, etc in healthy individuals.[45-50] Gaseous and particulate air pollutants with their soluble components may enter the lung and activate pulmonary neural reflexes and cause local inflammation to alter the autonomic nervous system and induce systemic inflammation.[51] Prior studies have reported that the cytokines associated with systemic inflammation such as interleukin-1ß, interleukin-6, tumor necrosis factor-alpha, intercellular adhesion molecule and C-reactive protein were significantly increased with exposure to ambient black carbon, nitrogen dioxide, PM_{10} and ozone.[45-50] Therefore, we hypothesize that the release of these cytokines following the exposure to pollutants activated inducible NOS.[41] Inducible NOS may have also been stimulated via activation of toll-like receptors (Tlr), especially Tlr2 and Tlr4.[52, 53] Our hypothesis is further supported by an animal study which reported activation of iNOS within 2 days of exposure to PM in ozone-induced rats.[54] In addition to iNOS stimulation, endothelial NOS (eNOS) stimulation as an injury response following endothelial membrane damage by pollutants may also be responsible for NO production. Although eNOS stimulation, not iNOS, has been shown in isolated rat aorta following acute exposure to sulphur dioxide[55], in our opinion, it is more likely that

iNOS is responsible for the observed increase in NO in the present study. This may be due to the fact that we observed a response in the first 24 hours for $PM_{2.5}$ and CO and not in the time lags shorter than 24 hours. This indicated that the response was probably not instantaneous. However, one may argue that greater degrees of exposure error and downward bias with shorter averaging times (for example, 3, 6 and 12 hours rather than 24 hours) may have resulted in the underestimation of the changes in plasma nitrite associated with pollutant concentrations over shorter time lags. Further research is needed to determine if the lack of increase in plasma nitrite observed in the shorter averaging times was a result of lack of sufficient time required for iNOS stimulation associated with shorter time frames or whether it was due to greater degrees of exposure error. Although an increase in the messenger RNA responsible for the production of iNOS can be seen as early as 3 hours following stimulation, a peak production of iNOS is observed within 12 hours of stimulation.[41] This timeframe is consistent with the timeframes within which we observed an increase in plasma nitrite. Nevertheless, simultaneous stimulation of both isoforms may have contributed to the observed increase in nitrite.

Regardless of the enzyme responsible for its generation, once formed, NO is responsible for several biochemical reactions within cells. It activates soluble cyclic guanylate cyclase which leads to relaxation of vascular smooth muscle cells and vasodilation.[41] In addition, it also mediates for inhibition of platelet adherence, and aggregation, inhibition of neutrophil chemotaxis, and signal transduction in the central and peripheral nervous systems.[41] It also acts as an antimicrobial effector. The newly formed NO may be consumed by the products of oxidative stress, superoxides, and peroxides and it may not be exclusively available to produce relaxation of vascular

smooth muscle and/or vasodilation. In fact, the excess consumption of NO may result in a decrease in endothelial function. This particularly depressing effect of systemic inflammation on endothelial function has been previously described in humans.[56] As mentioned previously, three studies have reported similar effects, i.e. reduction in BAFMD following ambient air pollutant exposures, of which at least one report[23] supported an inflammatory pathway activation as an underlying mechanism responsible for the observed decrease in endothelial function. Therefore, the fact that we did not observe a significant increase in PWA (vasodilation) and PAT ratio (improvement of endothelial function) is consistent with these reports. Another viewpoint that could potentially explain this finding is that the EndoPAT device failed to capture the improvement of endothelial function produced by the newly formed NO, assuming there was an improvement in the first place. This may be due to a fact that PAT ratio calculation captures an endothelial response at 60-90 seconds following cuff-deflation. Since the time interval within which a peak response of endothelium to hyperemic stimulus is observed may vary from subject to subject, the device may fail to capture a peak response in some subjects.

Our study demonstrated that ambient temperature over the first 24 hours was associated with a significant decrease in plasma nitrite and a significant increase in PWA and PAT ratio. While our findings with respect to the PWA and the PAT ratio are consistent with prior reports [36, 37], the association between temperature and plasma nitrite is of importance and needs to be further explored. A study by Zeka et al showed that inflammatory markers were associated with pollutants, especially in obese individuals.[46] However, in our study, we did not observe larger increases in plasma nitrite associated

with increased concentrations of pollutants among overweight and obese subjects compared to the subjects with normal BMI.

Our study has several strengths. We used two novel endothelial function markers to examine the change in these outcomes associated with increases in ambient pollutant concentrations in the previous few days. Also, the study design of repeated measures per subject reduced the chance of confounding by time-independent covariates where each subject acted as their own control. Moreover, it was possible to explore the changes in the markers of endothelial function associated with pollutants using time lags extending up to preceding 7 days due to the availability of continuously recorded hourly pollutant concentrations. While interpreting the results from this study, it is important to note a few limitations. First, exposure misclassification is likely to occur as ambient measurements of air pollutants rather than personal exposure measurements were used to approximate each subject's exposure. This exposure misclassification or measurement error may have resulted in underestimation of the changes in plasma nitrite associated with increases in ozone and NO_2 concentrations. Moreover, a monitor closest to the Busch campus was assigned to each subject regardless of the time spent in that area by each subject. As subjects were generally young (range 18-30 years) and recruited on the Busch campus, we assumed that subjects either lived on or nearby campus. However, it is possible that subjects may have stayed at their parental residence or any other location away from the Busch campus at the time of their study participation. This misclassification is likely to be non-differential with respect to the outcome measurement, likely resulting in underestimation of the effects of air pollutants, consistent with lack of association for PWA and PAT ratio.. Second, the study estimated

the changes in three outcomes associated with increases in five pollutants averaged over 7 time lags. However, we did not make inferences based on statistical significance, but based on the patterns of changes in the outcomes observed across lag times. For example, a consistent increase in plasma nitrite was observed with an IQR increase in $PM_{2.5}$, CO, and SO_2 on lag days 1, 2 and 3 indicating pattern of changes. Therefore, Bonferroni correction to account for multiple comparisons may be overaggressive in this situation and hence, was not performed. Third, a percent change in plasma nitrite concentration following a hyperemic stimulus (for example, forearm occlusion for 5 minutes) from its baseline can also be used as a marker of endothelial function. However, we could not examine the change in this outcome associated with ambient levels of air pollutants due to logistical constraints in obtaining repeated venous samples within a short frame of time.

Regardless of these limitations, our data generally support the hypothesis that ambient air pollution is associated with systemic inflammation in humans. To our knowledge, this is the first study addressing the effects of short-term ambient air pollutant concentrations on plasma nitrite. Future studies are warranted to examine the changes in plasma nitrite concentration following a hyperemic stimulus from its baseline (pre-to post cuff deflation) associated with increases in ambient air pollutants as well as changes in plasma nitrate concentration as a marker of nitric oxide bioavailability.

Tables

Table 1. Description of the Selected Monitors for Pollutants and Weather Parameters

Parameter	Name of the Monitor	Location of the Monitor	Latitude and Longitude	Distance from EOHSI*
Carbon Monoxide	Perth Amboy	130 Smith Street, Perth Amboy, NJ 08861	40.508764 and -74.268083	~15 miles
Sulphur Dioxide	Perth Amboy	130 Smith Street, Perth Amboy, NJ 08861	40.508764 and -74.268083	~15 miles
Nitrogen Dioxide	Rutgers University	20 Ryders lane, East Brunswick, NJ 08816	40.462182 and -74.429439	~6 miles
Ozone	Rutgers University	20 Ryders lane, East Brunswick, NJ 08816	40.462182 and -74.429439	~6 miles
$PM_{2.5}$	New Brunswick	Log Cabin road, East Brunswick, NJ 08816	40.4728 and -74.4225	~6 miles
Temperature and Relative Humidity	Newark	Newark International Airport Monitoring Station New Jersey Turnpike, Elizabeth, NJ 07201	40.683 and -74.169	~27 miles

* The EOHSI is located at 170 Frelinghuysen Road, Piscataway, New Jersey 08854.
† WBAN number or a fixed weather station identifier.

Table 2. Baseline Characteristics of Study Participants

Characteristic	Mean ± SD	Missing Obs N (%)	Distribution of Characteristics						
			Min	5th	25th	Median	75th	95th	Maximum
N	52								
Age (in Years)	21.2 ± 3.0	0 (0)	18	18	19	20	22	27	30
Systolic BP (in mm Hg)	115.5 (11.8)	1 (1.9)	90	100	108	112	124	138	142
Diastolic BP (in mm Hg)	69.9 (8.9)	1 (1.9)	50	58	62	70	76	84	90
Weight (in pounds)	165.3 ± 37.0	2 (3.9)	100	107	141	162	184	232	266
Body mass index (Kg/m^2)	25.0 ± 4.7	2 (3.9)	17.9	18.9	21.5	24	28.4	32.8	41.3
Serum Cholesterol (mg/dL)	155.4 ± 31.5	0 (0)	93	113	136	152	175.5	212	264
HDL Cholesterol (mg/dL)	51.4 ± 12.8	0 (0)	26	32	43.5	49	59	78	84
LDL Cholesterol (mg/dL)	82.7 ± 28.6	0 (0)	25	39	63	83	102.5	137	152
Serum triglyceride (mg/dL)	106.4 ± 63.5	0 (0)	32	44	66	91.5	129	245	338
Gender (M:F)	41:11	0 (0)							
Race		2 (3.9)							
White	40 (80%)								
Black	1 (2%)								
Asian	9 (18%)								
Ethnicity		1 (1.9)							
Hispanic	8 (15.7%)								
Non-Hispanic	43 (84.3%)								

Table 3. Distribution of Pollutant Concentrations and Meteorological Characteristics during the Study Period

Parameter*	No. of non-missing hours /days	Number of missing hours/days (% of Total hrs/days)	Inter quartile Range (IQR)	Mean ± SD	Distribution of Pollutant/Weather Parameter						
					Minimum	5th percentile	25th percentile	50th percentile	75th percentile	95th percentile	Maximum
$PM_{2.5}$ ($\mu g/m^3$)†											
Hourly	23,910	930 (3.7)	8.3	8.3 ± 7.7	0.0	0.2	3.0	6.2	11.3	23.9	70.3
Daily	989	46 (4.4)	7.0	8.2 ± 6.0	0.2	1.6	3.9	6.6	10.9	21.3	39.2
NO_2 (ppb)†											
Hourly	24,457	383 (1.5)	13.0	12.9 ± 9.9	0	2	5	10	18	33	74
Daily	1,027	8 (0.8)	9.6	13.0 ± 7.1	0.8	4.0	7.5	11.5	17.1	27.0	39.0
CO (ppm)†											
Hourly	23,906	934 (3.8)	0.2	0.35 ± 0.20	0	0.1	0.2	0.3	0.4	0.70	3.30
Daily	999	36 (3.5)	0.16	0.35 ± 0.13	0	0.17	0.26	0.33	0.42	0.59	0.93
SO_2 (ppb)†											
Hourly	24,201	639 (2.6)	2.0	2.39 ± 2.87	0	0	1	1	3	8	59
Daily	1,016	19 (1.8)	2.2	2.40 ± 2.03	0	0.1	1.0	1.9	3.2	6.7	13.8
O_3 (ppb)†											
Hourly	24,357	483 (1.9)	25	25.3 ± 17.9	2	2	11	24	36	58	114
Daily	1,027	8 (0.8)	17.1	25.3 ± 12.0	2	6.5	16.1	24.8	33.2	46.0	67.7
Temperature (Deg F)											
Hourly	24,838	2 (0.01)	29.2	54.4 ± 18.0	5	26.1	39.9	54	69.1	82	100
Daily	1,035	0 (0)	28.9	54.4 ± 17.2	11.3	26.8	40.5	54.5	69.4	79.7	91.6
Relative Humidity (%)											
Hourly	24,836	4 (0.02)	30	58.0 ± 19.0	5	28	43	58	73	88	100
Daily	1,035	0 (0)	20.9	58.0 ± 14.4	17.7	35.6	47.3	57.5	68.2	82.1	93.0

* Concentrations were measured hourly during the study period. Total possible hours =24,840, Total possible days=1,035

† $PM_{2.5}$, particulate matter less than 2.5 μm in aerodynamic diameter; ppb, parts per billion; ppm, parts per million

Table 4. Distribution of Daily Pollutant Concentrations and Meteorological Characteristics during the Study Period by Season

Parameter*	Season	No. of non-missing days‡	Number of missing days (% of Total days)‡	Mean ± SD	Distribution of Pollutant/Weather Parameter						
					Min	5th percentile	25th percentile	50th percentile	75th percentile	95th percentile	Max
$PM_{2.5}$ (μg/m^3)†	Overall	989	46 (4.4)	8.2 ± 6.0	0.2	1.6	3.9	6.6	10.9	21.3	39.2
	Spring	355	6 (1.7)	7.2 ± 5.1	0.3	1.4	3.5	5.7	9.7	17.8	25.1
	Summer	188	27 (12.6)	11.8 ± 8.1	0.7	2.4	5.4	9.3	16.5	27.5	39.2
	Fall	182	1 (0.6)	7.1 ± 5.0	0.2	1.5	3.4	5.6	10.1	17.0	26.8
	Winter	264	12 (4.4)	7.8 ± 5.1	0.7	1.9	4.0	7.0	10.3	17.0	31.1
NO_2 (ppb)†	Overall	1,027	8 (0.8)	13.0 ± 7.1	0.8	4.0	7.5	11.5	17.1	27.0	39.0
	Spring	361	0 (0)	13.1 ± 7.3	1.3	4.1	7.5	11.7	17.2	27.2	39.0
	Summer	211	4 (1.9)	8.7 ± 4.5	0.8	3.0	5.2	7.8	11.8	17.8	22.8
	Fall	183	0 (0)	11.2 ± 4.8	1.9	4.5	7.7	10.8	14.1	19.5	30.1
	Winter	272	4 (1.5)	17.2 ± 7.4	2.1	6.4	11.0	16.9	22.7	29.8	36.6
CO (ppm)†	Overall	999	36 (3.5)	0.35 ± 0.13	0	0.17	0.26	0.33	0.42	0.59	0.93
	Spring	351	10 (2.8)	0.34 ± 0.11	0.07	0.19	0.25	0.33	0.41	0.50	0.89
	Summer	203	12 (5.6)	0.36 ± 0.09	0.17	0.24	0.29	0.35	0.42	0.50	0.70
	Fall	174	9 (4.9)	0.35 ± 0.12	0.09	0.17	0.27	0.34	0.42	0.57	0.72
	Winter	271	5 (1.8)	0.35 ± 0.17	0	0.14	0.23	0.31	0.45	0.67	0.93
SO_2 (ppb)†	Overall	1,016	19 (1.8)	2.40 ± 2.03	0	0.1	1.0	1.9	3.2	6.7	13.8
	Spring	351	10 (2.8)	2.1 ± 1.8	0	0.08	0.88	1.7	2.8	5.5	10.3
	Summer	211	4 (1.9)	1.9 ± 1.5	0	0.3	0.9	1.6	2.5	4.7	9.0
	Fall	183	0 (0)	1.8 ± 1.4	0	0	0.8	1.5	2.5	4.4	7.3
	Winter	271	3 (1.1)	3.6 ± 2.6	0	0.7	1.5	2.8	4.9	8.4	13.8
O_3 (ppb)†	Overall	1,027	8 (0.8)	25.3 ± 12.0	2	6.5	16.1	24.8	33.2	46.0	67.7
	Spring	360	1 (0.3)	28.8 ± 9.8	2.8	11.1	22.7	29.2	34.7	44.5	60.9
	Summer	212	3 (0)	36.1 ± 10.9	5.8	19.0	29.2	35.7	43.0	54.0	67.7
	Fall	183	0 (0)	21.1 ± 8.2	5.5	11.1	15.2	19.4	26.7	34.1	51.3
	Winter	272	4 (1.5)	15.0 ± 7.3	2.0	3.3	9.1	15.1	20.2	27.2	33.2
Temperature (Deg F)	Overall	1,035	0 (0)	54.4 ± 17.2	11.3	26.8	40.5	54.5	69.4	79.7	91.6
	Spring	361	0 (0)	48.1 ± 14.0	14.7	24.0	38.4	48.4	58.2	70.8	79.8
	Summer	215	0 (0)	75.6 ± 5.5	60.4	66.3	72.3	75.8	78.8	85.2	91.6
	Fall	183	0 (0)	63.5 ± 9.0	41.4	47.2	56.8	65.0	69.9	76.6	80.9
	Winter	276	0 (0)	40.1 ± 10.5	11.3	23.8	32.8	39.5	47.0	58.9	66.8
Relative Humidity (%)	Overall	1,035	0 (0)	58.0 ± 14.4	17.7	35.6	47.3	57.5	68.2	82.1	93.0
	Spring	361	0 (0)	53.5 ± 16.0	17.7	30.3	41	52	66.5	80.9	89.3
	Summer	215	0 (0)	59.0 ± 11.5	35.7	41.5	50.3	58.4	66.5	78.4	87.8
	Fall	183	0 (0)	63.0 ± 11.1	39.1	46	54.2	62.7	70.3	82.8	90.3
	Winter	276	0 (0)	59.7 ± 14.5	28	38.4	48.3	57.3	72.9	84.5	93

Table 5. Pearson Correlation Coefficients for Daily‡ Pollutant Concentrations and Weather Parameters

Pollutant/Weather	Season	$PM_{2.5}$	CO	O_3	SO_2	NO_2	Temperature
$PM_{2.5}$	Overall						
CO		0.45					
O_3		0.19	-0.25				
SO_2		0.42	0.35	-0.26			
NO_2		0.23	0.51	-0.64	0.53		
Temperature		0.29	0.11	0.51	-0.27	-0.44	
Relative Humidity		0.16	0.29	-0.29	-0.11	0.12	0.19
$PM_{2.5}$	Spring						
CO		0.48					
O_3		-0.17	-0.47				
SO_2		0.54	0.34	-0.31			
NO_2		0.48	0.61	-0.67	0.51		
Temperature		0.20	0.06	0.44	-0.24	-0.26	
Relative Humidity		0.17	0.29	-0.40	-0.06	0.17	0.10
$PM_{2.5}$	Summer						
CO		0.44					
O_3		0.65	0.11				
SO_2		0.40	0.22	0.47			
NO_2		-0.02	0.45	-0.34	0.10		
Temperature		0.56	0.15	0.67	0.38	-0.26	
Relative Humidity		0.13	0.31	-0.25	-0.22	0.17	-0.25
$PM_{2.5}$	Fall						
CO		0.45					
O_3		0.52	-0.09				
SO_2		0.45	0.40	0.15			
NO_2		0.15	0.52	-0.47	0.37		
Temperature		0.45	0.10	0.53	-0.03	-0.21	
Relative Humidity		0.15	0.20	0.00	-0.19	0.00	0.37
$PM_{2.5}$	Winter						
CO		0.55					
O_3		-0.56	-0.66				
SO_2		0.64	0.42	-0.46			
NO_2		0.60	0.61	-0.80	0.58		
Temperature		0.03	0.26	-0.13	-0.15	-0.13	
Relative Humidity		0.19	0.33	-0.37	-0.15	0.15	0.52

‡ In instances where less than 75% of the hourly measurements (less than 18 of the 24) were available, an average for that day was set to missing.

Table 6. Distribution of Plasma Nitrite, Pulse Wave Amplitude (PWA) and PAT Ratio

Outcome	N	Mean ± SD	Distribution of Outcome						
			Minimum	5th percentile	25th percentile	50th percentile	75th percentile	95th percentile	Maximum
Plasma Nitrite (nM)	128*	130.8 ± 60.7	50.0	68.0	90.9	115.0	154.0	243.4	427.2
PAT Ratio†	138*	1.64 ± 0.45	1.00	1.12	1.36	1.51	1.75	2.70	3.33
PWA†	138*	573.8 ± 402.6	27.2	84.7	190.8	487.0	924.8	1261.6	1640.8

nM, nanomolars; * Missing data; † Unitless quanity

Table 7. Change in Plasma Nitrite, PWA and PAT Ratio associated with an IQR Increase in the Mean Temperature and Relative Humidity Lag Hours

Lag period (in hours)	Change in outcome associated with an IQR increase in mean temperature			Change in outcome associated with an IQR increase in mean relative humidity		
	Plasma Nitrite†	95% CI	p-value	Plasma Nitrite †	95% CI	p-value
0-23	-29.3	-49.7, -9.0	***0.0053***	-8.9	-22.2, 4.5	0.19
24-47	-27.3	-47.7, -6.9	***0.0095***	-5.5	-18.1, 7.0	0.38
48-71	-22.9	-43.8, -2.0	***0.033***	-8.5	-22.1, 5.1	0.22
72-95	-14.6	-35.4, 6.2	0.17	-6.2	-18.2, 5.7	0.30
96-119	-8.4	-29.4, 12.5	0.43	-9.9	-22.1, 2.3	0.11
120-143	-14.6	-35.5, 6.4	0.17	2.9	-9.9, 15.7	0.65
144-167	-16.1	-37.6, 5.4	0.14	2.5	-10.4, 15.3	0.70
Lag period	PAT Ratio†	95%CI	p-value	PAT Ratio†	95%CI	p-value
0-23	0.17	0.03, 0.32	***0.021***	-0.05	-0.16, 0.05	0.33
24-47	0.17	0.03, 0.32	***0.022***	-0.09	-0.19, 0.006	0.070
48-71	0.12	-0.03, 0.27	0.13	-0.03	-0.14, 0.080	0.54
72-95	0.12	-0.03, 0.27	0.10	0.03	-0.07, 0.12	0.60
96-119	0.10	-0.05, 0.25	0.20	0.009	-0.09, 0.11	0.85
120-143	0.08	-0.07, 0.23	0.29	-0.03	-0.14, 0.07	0.52
144-167	0.06	-0.10, 0.21	0.46	-0.10	-0.20, 0.004	0.060
Lag period	PWA†	95%CI	p-value	PWA †	95%CI	p-value
0-23	155.4	22.7, 288.0	***0.022***	60.3	-14.8, 135.4	0.11
24-47	56.7	-75.1, 188.4	0.39	-14.8	-85.9, 56.4	0.68
48-71	91.2	-47.8, 230.3	0.20	-47.7	-125.6, 30.2	0.23
72-95	112.3	-20.4, 245.1	0.096	9.3	-57.8, 76.3	0.78
96-119	129.2	-2.0, 260.5	0.054	-3.5	-72.8, 65.7	0.92
120-143	95.5	-33.4, 224.3	0.14	-3.6	-78.3, 71.0	0.92
144-167	48.2	-83.9, 180.4	0.47	-30.6	-103.5, 42.3	0.41

Table 8. Change in Plasma Nitrite, PWA and PAT Ratio associated with Calendar Month, Day of the Week, and Year

Confounder	Change in Plasma Nitrite (95%CI)	p-value	Change in PAT Ratio (95%CI)	p-value	Change in PWA ¶ (95%CI)	p-value
Calender Month		0.40†		***0.013***†		0.20†
Jan vs Dec	-23.3 (-91.6, 45.1)	0.48	-0.02 (-0.49, 0.46)	0.93	-199.6 (-596.9, 197.8)	0.31
Feb vs Dec	14.1 (-58.4, 86.5)	0.69	-0.09 (-0.52, 0.33)	0.65	-522.2 (-947.6, -96.8)	***0.019***
Mar vs Dec	46.4 (-18.3, 111.1)	0.15	0.70 (0.33, 1.08)	***0.0009***	-33.1 (-409.0, 342.9)	0.86
Apr vs Dec	2.8 (-57.6, 63.2)	0.92	0.16 (-0.21, 0.54)	0.37	-22.0 (-382.0, 337.9)	0.90
May vs Dec	-4.4 (-63.4, 54.6)	0.88	0.42 (0.06, 0.79)	***0.025***	-223.9 (-576.0, 128.2)	0.20
Jun vs Dec	-7.2 (-67.0, 52.7)	0.80	0.24 (-0.13, 0.61)	0.19	32.8 (-325.3, 391.0)	0.85
Jul vs Dec	-24.3 (-93.1, 44.5)	0.47	0.04 (-0.41, 0.49)	0.86	21.0 (-390.0, 432.0)	0.92
Aug vs Dec	-3.3 (-75.9, 69.2)	0.92	0.39 (-0.05, 0.83)	0.079	-34.1 (-477.3, 409.1)	0.87
Sept vs Dec	32.7 (-32.7, 98.0)	0.31	0.40 (0.03, 0.76)	***0.035***	71.1 (-298.9, 441.0)	0.69
Oct vs Dec	-4.6 (-64.6, 55.4)	0.87	0.17 (-0.21, 0.55)	0.36	-17.6 (-364.6, 329.4)	0.92
Nov vs Dec	27.1 (-24.5, 78.7)	0.28	0.10 (-0.24, 0.45)	0.54	-43.9 (-319.8, 232.0)	0.74
Day of the Week		0.53†		0.75†		0.55†
Mon. vs Friday	-21.1 (-111.6, 69.5)	0.58	-0.20 (-0.81, 0.41)	0.45	171.8 (-370.7, 714.4)	0.48
Tues. vs Friday	-34.1 (-96.5, 28.2)	0.22	0.18 (-0.26, 0.61)	0.36	68.5 (-289.8, 426.8)	0.66
Wed. vs Friday	-6.5 (-44.2, 31.3)	0.68	-0.01 (-0.25, 0.22)	0.89	98.1 (-131.2, 327.3)	0.35
Thurs. vs Friday	-30.4 (-87.8, 27.0)	0.23	0.03 (-0.32, 0.37)	0.86	207.4 (-72.5, 487.3)	0.55
Year		***0.0028***†		0.89†		0.094†
2006 vs 2009	78.0 (15.3, 140.7)	***0.015***	0.14 (-0.29, 0.57)	0.52	421.0 (-7.5, 849.4)	0.054
2007 vs 2009	45.0 (-6.02, 96.0)	0.083	0.15 (-0.23, 0.54)	0.44	432.2 (54.5, 810.0)	***0.025***
2008 vs 2009	5.9 (-45.8, 57.5)	0.82	0.15 (-0.24, 0.54)	0.45	493.8 (111.7, 875.9)	0.094

† Overall p-values were obtained from type 3 tests of fixed effects.

Table 9. Changes in Plasma Nitrite, PWA and PAT Ratio Associated with an Interquartile Range Increase in the Mean Pollutant concentrations in Single Pollutant Mixed-Effects Models

Outcome	Lag period (in hours)	$PM_{2.5}$ (µg/m3) IQR:7.0 µg/m3		Ozone (ppb) IQR: 17.1 ppb		NO_2 (ppb) IQR: 9.6 ppb		SO_2 (ppb) IQR: 2.2 ppb		CO (ppb) IQR: 161.7 ppb	
		Change in Outcome (95%CI)	p-Value	Change in Outcome (95%CI)	p-Value	Change in Outcome (95%CI)	p-Value	Change in Outcome (95%CI)	p-Value	Change in Outcome (95%CI)	p-Value
Plasma Nitrite	0-23	***15.5 (2.4, 28.5)***	***0.021***	-6.4 (-23.5, 10.8)	0.46	3.5 (-14.5, 21.4)	0.70	11.8 (-1.0, 24.5)	0.070	***15.6 (2.4, 28.9)***	***0.021***
	24-47	7.9 (-3.7, 19.6)	0.18	-5.6 (-20.9, 9.6)	0.47	11.8 (-2.6, 26.2)	0.11	9.9 (-0.2, 20.0)	0.054	11.6 (-2.0, 25.3)	0.095
	48-71	6.2 (-4.8, 17.1)	0.27	-1.8 (-18.1, 14.4)	0.82	6.3 (-8.8, 21.4)	0.41	10.6 (-2.9, 24.2)	0.12	6.8 (-6.1, 19.7)	0.30
	72-95	-2.0 (-11.9, 7.9)	0.69	***14.4 (1.0, 27.7)***	***0.035***	-15.6 (-31.9, 0.70)	0.060	-4.1 (-14.3, 6.1)	0.43	-0.2 (-14.0, 13.7)	0.98
	96-119	4.8 (-6.7, 16.3)	0.41	-0.2 (-15.7, 15.3)	0.98	9.6 (-3.7, 22.9)	0.15	***14.3 (3.4, 25.3)***	***0.011***	4.6 (-9.0, 18.2)	0.50
	120-143	-6.1 (-16.1, 3.9)	0.23	-11.8 (-27.5, 3.8)	0.14	2.4 (-12.3, 17.1)	0.75	-8.9 (-24.9, 7.0)	0.27	2.5 (-11.4, 16.4)	0.72
	144-167	-4.5 (-14.4, 5.4)	0.37	-13.0 (-26.8, 0.7)	0.062	7.9 (-6.7, 22.5)	0.28	9.3 (-4.8, 23.4)	0.19	5.6 (-6.1, 17.3)	0.35
PAT Ratio	0-23	-0.09 (-0.19, 0.01)	0.086	-0.05 (-0.19, 0.10)	0.51	-0.04 (-0.18, 0.10)	0.60	-0.09 (-0.20, 0.02)	0.12	-0.06 (-0.17, 0.05)	0.31
	24-47	0.01 (-0.08, 0.10)	0.83	0.07 (-0.06, 0.21)	0.28	-0.02 (-0.15, 0.10)	0.72	-0.04 (-0.12, 0.05)	0.38	0.03 (-0.09, 0.14)	0.61
	48-71	0.02 (-0.07, 0.11)	0.69	-0.01 (-0.14, 0.12)	0.86	-0.02 (-0.14, 0.10)	0.78	-0.04 (-0.14, 0.05)	0.35	0.06 (-0.05, 0.16)	0.28
	72-95	0.03 (-0.06, 0.11)	0.54	0.01 (-0.11, 0.13)	0.85	0.02 (-0.11, 0.16)	0.73	-0.03 (-0.11, 0.05)	0.49	0.06 (-0.07, 0.18)	0.36
	96-119	0.03 (-0.06, 0.12)	0.50	0.004 (-0.12, 0.13)	0.96	-0.05 (-0.17, 0.06)	0.34	-0.04 (-0.14, 0.06)	0.42	0.02 (-0.09, 0.13)	0.75
	120-143	0.001 (-0.09, 0.09)	0.98	0.12 (-0.009, 0.25)	0.070	-0.11 (-0.23, 0.01)	0.080	-0.06 (-0.17, 0.06)	0.36	0.005 (-0.11, 0.12)	0.93
	144-167	0.03 (-0.05, 0.11)	0.50	0.03 (-0.09, 0.15)	0.68	-0.02 (-0.15, 0.11)	0.78	0.04 (-0.08, 0.16)	0.47	0.03 (-0.07, 0.12)	0.62
PWA	0-23	33.5 (-46.8, 113.8)	0.41	4.2 (-96.2, 104.7)	0.93	38.4 (-61.6, 138.4)	0.45	1.7 (-78.7, 82.2)	0.97	27.8 (-53.2, 108.8)	0.50
	24-47	-22.3 (-88.2, 43.6)	0.50	-12.5 (-105.4, 80.3)	0.79	5.0 (-82.6, 92.6)	0.91	-42.7 (-104.3, 19.0)	0.17	-14.2 (-95.6, 67.2)	0.73
	48-71	1.4 (-61.6, 64.4)	0.97	18.5 (-82.3, 119.3)	0.72	24.7 (-64.1, 113.5)	0.58	21.7 (-45.7, 89.2)	0.52	30.0 (-43.4, 103.4)	0.42
	72-95	22.8 (-37.2, 82.8)	0.45	-15.0 (-98.9, 68.8)	0.72	-6.6 (-105.7, 92.5)	0.90	1.3 (-55.1, 57.7)	0.96	8.2 (-76.7, 93.2)	0.85
	96-119	3.9 (-60.8, 68.5)	0.91	-18.2 (-110.8, 74.4)	0.70	31.0 (-52.2, 114.2)	0.46	30.6 (-38.8, 100.1)	0.38	29.1 (-52.3, 110.5)	0.48
	120-143	-4.8 (-65.7, 56.2)	0.88	18.7 (-76.2, 113.5)	0.70	-49.7 (-137.0, 37.6)	0.26	14.3 (-69.4, 97.9)	0.74	-36.1 (-112.7, 40.5)	0.35
	144-167	-24.9 (-84.3, 34.6)	0.41	12.4 (-73.7, 98.4)	0.78	-28.9 (-119.1, 61.2)	0.53	-78.9 (-164.9, 7.0)	0.071	-30.6 (-104.1, 43.0)	0.41

Table 10. Association between plasma nitrite and pollutants stratified by BMI

Outcome	$PM_{2.5}$ (µg/m3) (Lag 0-23) IQR:6.97 µg/m3		CO (ppb) (Lag 0-23) IQR: 161.7 ppb	
Plasma Nitrite				
All	15.5 (2.4, 28.5)	0.021	15.6 (2.4, 28.9)	0.021
Normal: (BMI <25) , N=28 subjects	24.3 (4.6, 44.0)	0.017	11.5 (-7.0, 29.9)	0.22
Overweight/Obese (BMI: ≥ 25), N=22 subjects	8.2 (-10.1, 26.5)	0.37	0.64 (-19.8, 21.0)	0.95
Plasma Nitrite	SO_2 (ppb) (Lag 96-119) IQR: 2.17 ppb		Ozone (ppb) (Lag 72-95) IQR: 17.08 ppb	
All	14.3 (3.4, 25.3)	0.011	14.4 (1.0, 27.7)	0.035
Normal: (BMI <25)	15.7 (-1.4, 32.9)	0.071	22.9 (5.3, 40.4)	0.012
Overweight/Obese (BMI: ≥ 25)	2.9 (-13.3, 19.1)	0.72	7.0 (-12.5, 26.4)	0.47

BMI missing info on 2 subjects of the total 52.

Table 11. Changes in Plasma Nitrite, PWA and PAT Ratio Associated with an Interquartile Range Increase in the Mean Pollutant concentration Lag Hours (adjusted for respective temperature lags)

Outcome	Lag period (in hours)	$PM_{2.5}$ (µg/m3) IQR:7.0 µg/m3		Ozone (ppb) IQR: 17.1 ppb		NO_2 (ppb) IQR: 9.6 ppb		SO_2 (ppb) IQR: 2.2 ppb		CO (ppb) IQR: 161.7 ppb	
		Change in Outcome (95%CI)	p-Value	Change in Outcome (95%CI)	p-Value	Change in Outcome (95%CI)	p-Value	Change in Outcome (95%CI)	p-Value	Change in Outcome (95%CI)	p-Value
Plasma Nitrite	0-23	***15.5 (2.4, 28.5)***	***0.021***	-6.4 (-23.5, 10.8)	0.46	3.5 (-14.5, 21.4)	0.70	11.8 (-1.0, 24.5)	0.070	***15.6 (2.4, 28.9)***	***0.021***
	24-47	7.3 (-4.8, 19.3)	0.23	3.7 (-13.2, 20.6)	0.66	2.3 (-12.4, 17.0)	0.76	7.4 (-2.7, 17.6)	0.15	5.7 (-7.8, 19.2)	0.40
	48-71	10.8 (-2.1, 23.8)	0.10	8.7 (-9.3, 26.7)	0.34	-0.8 (-16.0, 14.4)	0.91	14.0 (-0.7, 28.7)	0.062	4.7 (-8.8, 18.1)	0.49
	72-95	-4.4 (-16.8, 8.0)	0.48	***24.6 (9.5, 39.6)***	***0.0016***	***-24.5 (-40.6, -8.5)***	***0.0031***	-5.8 (-17.2, 5.6)	0.31	-3.3 (-18.4, 11.9)	0.67
	96-119	0.5 (-12.7, 13.7)	0.94	2.7 (-14.5, 19.9)	0.75	3.0 (-11.0, 16.9)	0.68	8.9 (-3.5, 21.3)	0.16	4.6 (-10.2, 19.5)	0.54
	120-143	-8.7 (-19.8, 2.5)	0.13	-10.7 (-28.5, 7.1)	0.24	1.3 (-14.7, 17.4)	0.87	-14.1 (-31.7, 3.5)	0.12	0.4 (-14.9, 15.7)	0.96
	144-167	-0.5 (-12.6, 11.6)	0.94	***-15.9 (-30.7, -1.0)***	***0.037***	11.5 (-4.0, 27.0)	0.14	14.3 (-1.7, 30.3)	0.080	8.3 (-4.5, 21.0)	0.20
PAT Ratio	0-23	-0.09 (-0.19, 0.01)	0.086	-0.05 (-0.19, 0.10)	0.51	-0.04 (-0.18, 0.10)	0.60	-0.09 (-0.20, 0.02)	0.12	-0.06 (-0.17, 0.05)	0.31
	24-47	0.006 (-0.09, 0.10)	0.90	0.05 (-0.10, 0.19)	0.52	0.002 (-0.12, 0.12)	0.97	-0.04 (-0.12, 0.04)	0.32	0.043 (-0.07, 0.15)	0.48
	48-71	0.04 (-0.06, 0.14)	0.41	-0.05 (-0.19, 0.09)	0.50	0.02 (-0.10, 0.13)	0.79	-0.05 (-0.14, 0.05)	0.35	0.07 (-0.03, 0.17)	0.16
	72-95	0.03 (-0.06, 0.13)	0.47	-0.02 (-0.16, 0.11)	0.72	0.07 (-0.06, 0.20)	0.29	-0.02 (-0.11, 0.06)	0.58	0.07 (-0.06, 0.19)	0.29
	96-119	0.03 (-0.07, 0.13)	0.54	-0.01 (-0.15, 0.12)	0.85	-0.03 (-0.14, 0.08)	0.59	-0.03 (-0.13, 0.07)	0.60	0.02 (-0.10, 0.13)	0.78
	120-143	0.02 (-0.08, 0.11)	0.70	***0.15 0.01, 0.29)***	***0.034***	-0.11 (-0.234, 0.01)	0.080	-0.04 (-0.16, 0.08)	0.50	0.008 (-0.11, 0.12)	0.88
	144-167	0.05 (-0.04, 0.14)	0.26	0.05 (-0.07, 0.18)	0.39	-0.04 (-0.17, 0.09)	0.52	0.05 (-0.08, 0.18)	0.43	0.02 (-0.08, 0.13)	0.64
PWA	0-23	33.5 (-46.8, 113.8)	0.41	4.2 (-96.2, 104.7)	0.93	38.4 (-61.6, 138.4)	0.45	1.7 (-78.7, 82.2)	0.97	27.8 (-53.2, 108.8)	0.50
	24-47	4.6 (-64.9, 74.1)	0.90	-10.5 (-116.3, 95.2)	0.84	26.3 (-63.0, 115.7)	0.56	-20.6 (-83.2, 42.0)	0.52	8.4 (-73.0, 89.8)	0.84
	48-71	4.1 (-68.4, 76.6)	0.91	9.0 (-101.1, 119.1)	0.87	40.2 (-47.6, 128.1)	0.37	30.8 (-40.4, 102.0)	0.39	30.3 (-45.2, 105.8)	0.43
	72-95	17.8 (-54.3, 89.9)	0.62	- 53.4 (-144.7, 38.0)	0.25	18.0 (-78.4, 114.5)	0.71	2.1 (-57.6, 61.9)	0.94	9.5 (-79.9, 98.9)	0.83
	96-119	4.2 (-67.4, 75.8)	0.91	-45.8 (-143.2, 51.6)	0.35	56.3 (-25.0, 137.7)	0.17	39.3 (-32.1, 110.7)	0.28	24.8 (-59.7, 109.3)	0.56
	120-143	15.0 (-50.3, 80.2)	0.65	29.5 (-73.4, 132.4)	0.57	62.2 (-153.5, 29.1)	0.18	22.7 (-63.8, 109.2)	0.60	-33.6 (-114.6, 47.4)	0.41
	144-167	-26.9 (-96.1, 42.3)	0.44	39.8 (-50.2, 129.7)	0.38	-40/2 (-132.4, 51.9)	0.39	-81.0 (-174.8, 12.9)	0.090	-36.3 (-113.5, 40.8)	0.35

Table 12. Changes in Plasma Nitrite, PWA and PAT Ratio Associated with an Interquartile Range Increase in the Mean Pollutant concentration Lag Hours using cumulative lag hours

Outcome	Lag period (in hours)	$PM_{2.5}$ (µg/m3) IQR:6.97 µg/m3		Ozone (ppb) IQR: 17.08 ppb		NO_2 (ppb) IQR: 9.63 ppb		SO_2 (ppb) IQR: 2.17 ppb		CO (ppb) IQR: 161.7 ppb	
		Change in Outcome (95%CI)	p-Value	Change in Outcome (95%CI)	p-Value	Change in Outcome (95%CI)	p-Value	Change in Outcome (95%CI)	p-Value	Change in Outcome (95%CI)	p-Value
Plasma Nitrite	0-23	***15.5 (2.4, 28.5)***	***0.021***	-6.4 (-23.5, 10.8)	0.46	3.5 (-14.5, 21.4)	0.70	11.8 (-1.0, 24.5)	0.070	***15.6 (2.4, 28.9)***	***0.021***
	0-47	***16.8 (2.8, 30.8)***	***0.019***	-11.2 (-31.8, 9.4)	0.28	13.7 (-5.6, 32.9)	0.16	***17.0 (3.6, 30.4)***	***0.014***	***16.0 (2.9, 29.1)***	***0.017***
	0-71	***15.8 (1.9, 29.8)***	***0.027***	-8.9 (-30.5, 12.8)	0.42	15.8 (-3.8, 35.5)	0.11	***18.8 (4.4, 33.2)***	***0.011***	***13.1 (0.8, 25.4)***	***0.037***
	0-95	11.2 (-2.5, 24.9)	0.11	3.8 (-18.4, 26.1)	0.73	7.3 (-14.2, 28.7)	0.50	12.4 (-2.7, 27.5)	0.11	11.0 (-1.5, 23.5)	0.084
	0-119	10.8 (-2.7, 24.4)	0.12	3.2 (-20.4, 26.8)	0.79	10.8 (-7.9, 29.4)	0.25	***19.1 (3.9, 34.4)***	***0.014***	***10.7 (-1.8, 23.1)***	***0.092***
	0-143	4.8 (-8.8, 18.3)	0.49	-2.8 (-28.3, 22.7)	0.83	9.5 (-8.2, 27.1)	0.29	14.9 (-1.9, 31.6)	0.082	10.6 (-1.5, 22.6)	0.085
	0-167	2.4 (-11.5, 16.2)	0.73	-10.9 (-38.4, 16.7)	0.44	11.8 (-6.2, 29.9)	0.20	***19.2 (0.7, 37.6)***	***0.042***	***13.8 (0.4, 27.3)***	***0.044***
PAT Ratio	0-23	-0.09 (-0.19, 0.01)	0.086	-0.05 (-0.19, 0.10)	0.51	-0.04 (-0.18, 0.10)	0.60	-0.09 (-0.20, 0.02)	0.12	-0.06 (-0.17, 0.05)	0.31
	0-47	-0.04 (-0.15, 0.06)	0.41	0.02 (-0.16, 0.19)	0.85	-0.04 (-0.20, 0.12)	0.62	-0.09 (-0.21, 0.03)	0.13	-0.02 (-0.13, 0.09)	0.76
	0-71	-0.03 (-0.14, 0.07)	0.55	0.01 (-0.17, 0.19)	0.89	-0.04 (-0.20, 0.12)	0.59	-0.09 (-0.21, 0.02)	0.12	0.02 (-0.09, 0.12)	0.75
	0-95	-0.02 (-0.12, 0.09)	0.77	0.02 (-0.17, 0.20)	0.86	-0.03 (-0.20, 0.13)	0.70	-0.09 (-0.21, 0.02)	0.12	0.03 (-0.07, 0.14)	0.55
	0-119	-0.02 (-0.13, 0.09)	0.69	0.02 (-0.18, 0.22)	0.86	-0.06 (-0.21, 0.10)	0.47	-0.10 (-0.22, 0.02)	0.12	0.03 (-0.08, 0.14)	0.59
	0-143	0.01 (-1.0, 0.12)	0.83	0.08 (-0.14, 0.30)	0.47	-0.09 (-0.24, 0.06)	0.22	-0.12 (-0.25, 0.006)	0.061	0.03 (-0.08, 0.15)	0.57
	0-167	0.02 (-0.09, 0.13)	0.73	0.10 (-0.14, 0.33)	0.41	-0.10 (-0.26, 0.06)	0.21	-0.12 (-0.27, 0.02)	0.091	0.05 (-0.08, 0.17)	0.48
PWA	0-23	33.5 (-46.8, 113.8)	0.41	4.2 (-96.2, 104.7)	0.93	38.4 (-61.6, 138.4)	0.45	1.7 (-78.7, 82.2)	0.97	27.8 (-53.2, 108.8)	0.50
	0-47	-3.3 (-83.0, 76.4)	0.93	-6.9 (-125.9, 112.0)	0.91	19.6 (-91.3, 130.6)	0.73	-43.6 (-128.7, 41.4)	0.31	7.7 (-72.5, 88.0)	0.85
	0-71	-1.6 (-79.7, 76.4)	0.97	1.8 (-125.0, 128.6)	0.98	24.3 (-86.7, 135.4)	0.66	-17.2 (-101.9, 67.5)	0.69	20.0 (-54.3, 94.4)	0.59
	0-95	15.4 (-62.5, 93.3)	0.70	-10.0 (-141.2, 121.3)	0.88	45.3 (-78.1, 168.7)	0.47	-12.2 (-96.3, 71.8)	0.77	19.1 (-56.8, 95.0)	0.62
	0-119	14.1 (-64.8, 93.0)	0.72	-17.0 (-156.1, 122.0)	0.81	48.8 (-61.6, 159.3)	0.38	3.4 (-82.7, 89.5)	0.94	22.6 (-53.3, 98.5)	0.56
	0-143	-1.6 (-79.9, 76.7)	0.97	-8.2 (-162.3, 145.9)	0.92	20.3 (-85.2, 125.7)	0.70	0.5 (-91.6, 92.6)	0.99	10.1 (-69.0, 89.2)	0.80
	0-167	-9.7 (-91.3, 71.8)	0.81	-0.2 (-170.0, 169.7)	0.99	8.5 (-101.3, 118.3)	0.88	-20.8 (-125.1, 83.5)	0.69	2.0 (-87.2, 91.1)	0.97

Table 13. Effect of Pollutants on Plasma nitrite concentration: Two Pollutant Models

Lag Period (in hrs)	Single Pollutant Model Estimates (95% CI)	Two Pollutant Model # 1 Estimates (95% CI)	Two Pollutant Model # 2 Estimates (95% CI)	Two Pollutant Model # 3 Estimates (95% CI)	Two Pollutant Model # 4 Estimates (95% CI)	Two Pollutant Model # 5 Estimates (95% CI)	Two Pollutant Model # 6 Estimates (95% CI)
0-23							
$PM_{2.5}$	***15.5 (2.4, 28.5)***	12.8 (-1.2, 26.7)	***11.0 (-4.0, 25.9)***		***16.1 (3.0, 29.1)***		
SO_2	11.8 (-1.0, 24.5)	9.3 (-5.4, 24.0)		8.7 (-4.1, 21.6)			12.1 (-0.9, 25.1)
CO	***15.6 (2.4, 28.9)***		10.7 (-5.1, 26.5)	13.5 (-0.02, 27.0)		***15.5 (1.6, 29.3)***	
O_3	-6.4 (-23.5, 10.8)				-12.2 (-30.8, 6.4)	-0.04 (-17.0, 16.9)	-6.0 (-22.8, 10.7)
72-95							
$PM_{2.5}$	-2.0 (-11.9, 7.9)	2.6 (-9.4, 14.5)	-1.1 (-12.6, 10.5)		-5.8 (-16.2, 4.5)		
SO_2	-4.1 (-14.3, 6.1)	-8.4 (-21.4, 4.6)		-4.4 (-15.0, 6.2)			-6.0 (-16.2, 4.1)
CO	-0.2 (-14.0, 13.7)		-1.9 (-18.6, 14.7)	1.3 (-13.0, 15.6)		2.8 (-11.2, 16.9)	
O_3	***14.4 (1.0, 27.7)***				***16.9 (1.6, 32.3)***	13.6 (-0.7, 27.9)	***15.5 (2.0, 29.0)***
96-119							
$PM_{2.5}$	4.8 (-6.7, 16.3)	-3.6 (-17.6, 10.5)	5.1 (-8.6, 18.9)		4.9 (-6.7, 16.5)		
SO_2	***14.3 (3.4, 25.3)***	***16.0 (1.6, 30.5)***		***15.6 (3.1, 28.1)***			***14.5 (3.4,25.6)***
CO	4.6 (-9.0, 18.2)		-0.4 (-17.4, 16.6)	-4.0 (-19.1, 11.0)		5.2 (-9.1, 19.5)	
O_3	-0.2 (-15.7, 15.3)				-1.3 (-18.9, 16.3)	2.2 (-14.1, 18.4)	1.4 (-13.8, 16.7)

CI, Confidence Interval

Table 14. Assessment of Between and Within Subject Effects on Plasma Nitrite

Outcome	$PM_{2.5}$ (µg/m3) (Lag 0-23) IQR:6.97 µg/m3		CO (ppb) (Lag 0-23) IQR: 161.7 ppb	
Plasma Nitrite				
Combined	15.5 (2.4, 28.5)	0.021	15.6 (2.4, 28.9)	0.021
Between	2.7 (-24.2, 29.6)	0.84	7.2 (-26.1, 40.5)	0.66
Within	***19.6 (4.5, 34.7)***	***0.012***	***17.2 (3.0, 31.5)***	***0.019***
Plasma Nitrite	SO_2 (ppb) (Lag 96-119) IQR: 2.17 ppb		Ozone (ppb) (Lag 72-95) IQR: 17.08 ppb	
Combined	14.3 (3.4, 25.3)	0.011	14.4 (1.0, 27.7)	0.035
Between	***40.4 (13.4, 67.5***	***0.0045***	15.4 (-26.4, 57.1)	0.46
Within	10.5 (-0.83, 21.9)	0.070	***14.3 (0.04, 28.5)***	***0.049***

Figures

Figure 1. Mean Pollutant Concentrations by Hour of the Day

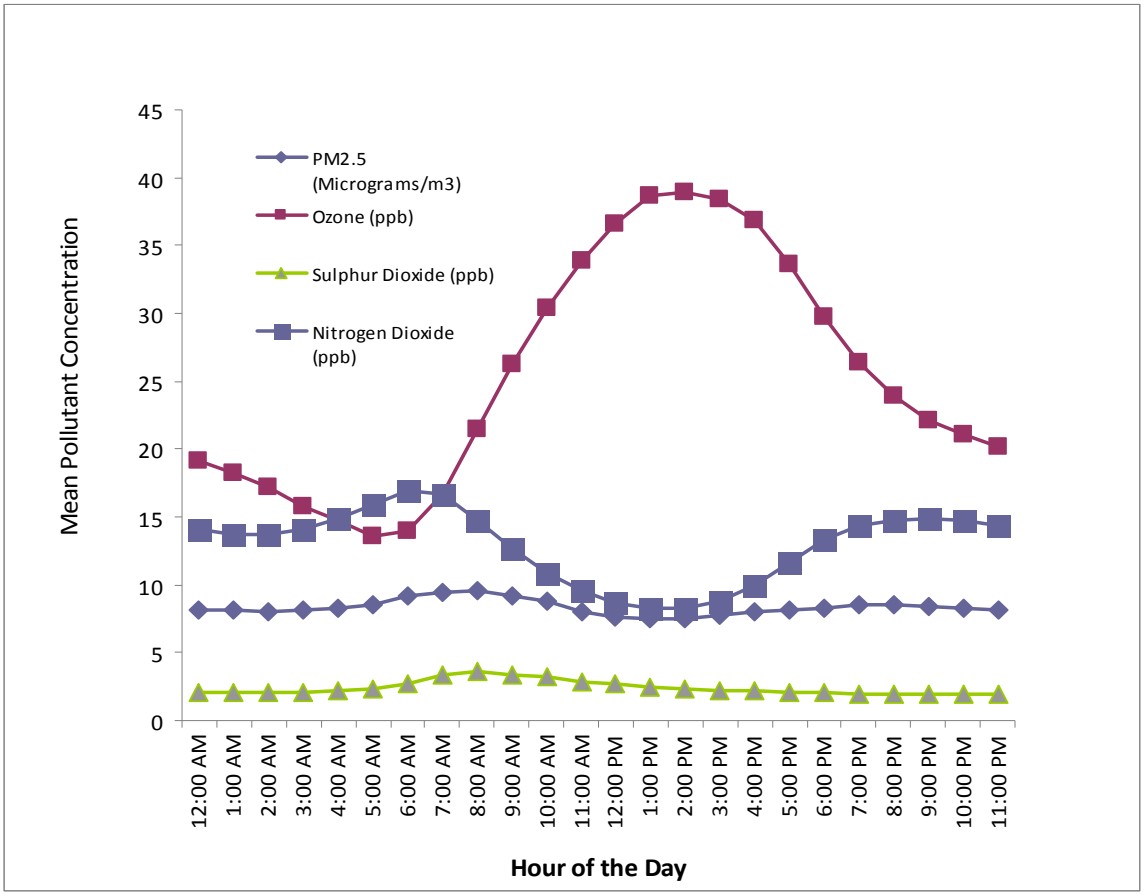


Figure 2. Mean Carbon Monoxide Concentration by Hour of the Day

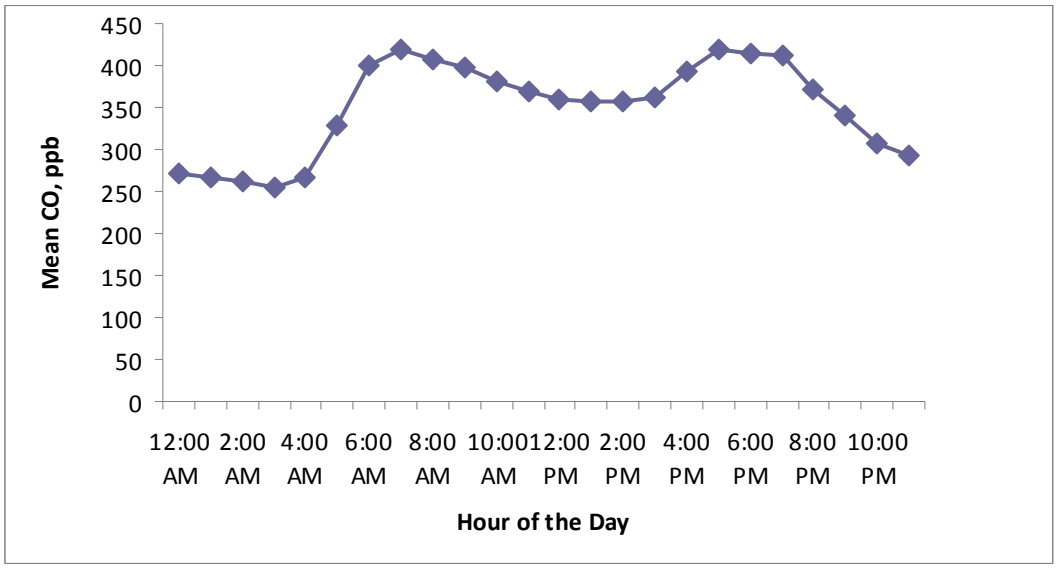


Figure 3. Mean Pollutant Concentrations by Day of the Week

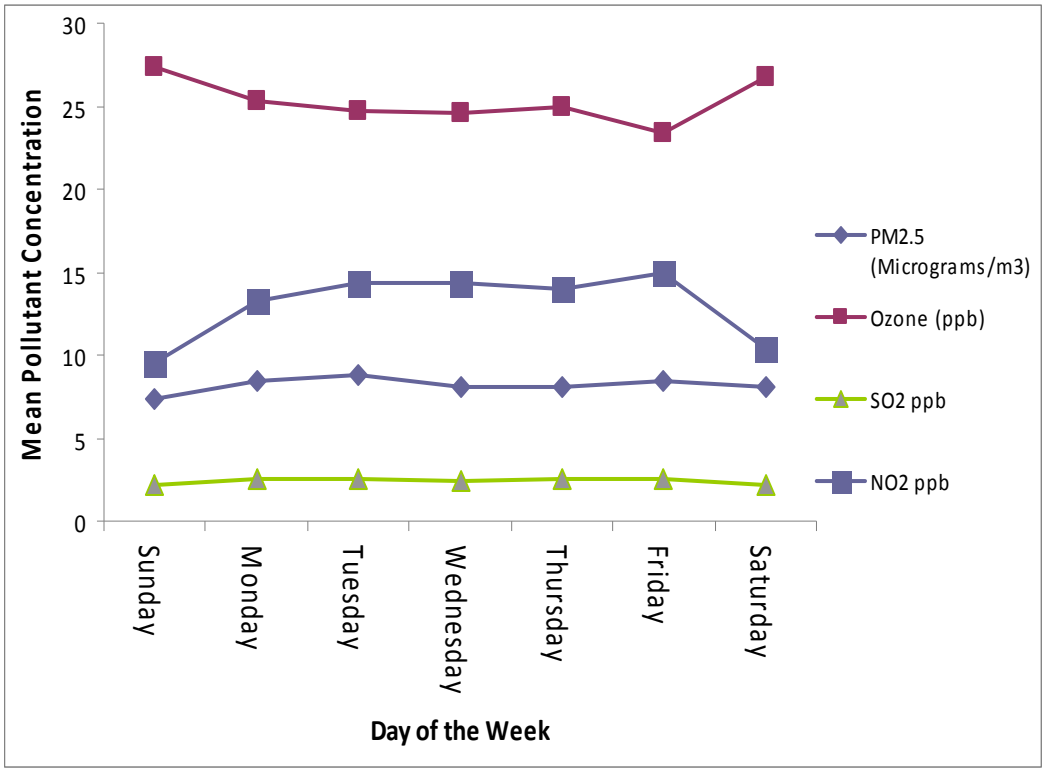


Figure 4. Mean Carbon Monoxide Concentration by Day of the Week

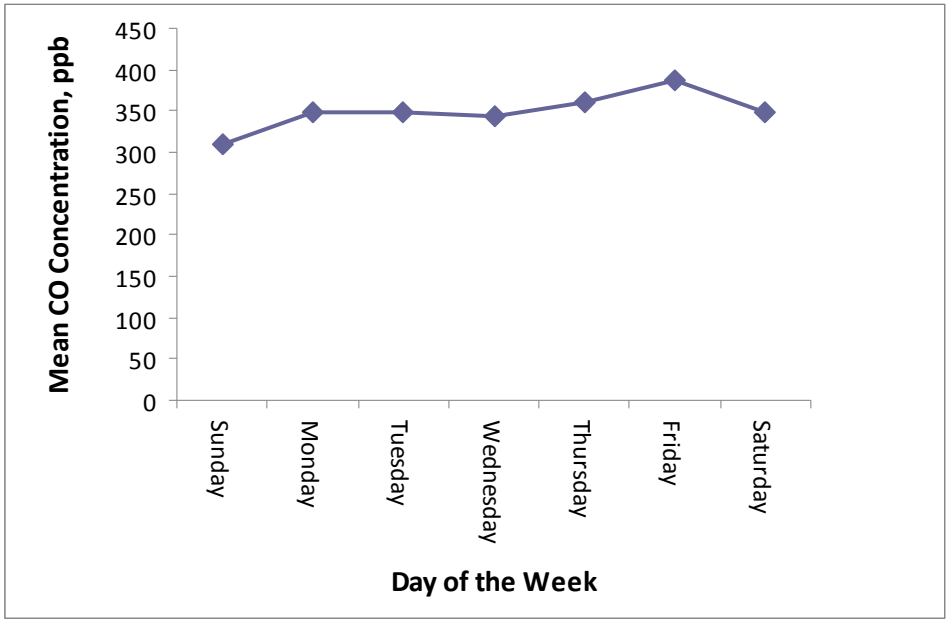


Figure 5. Mean Pollutant Concentrations by Calender Month

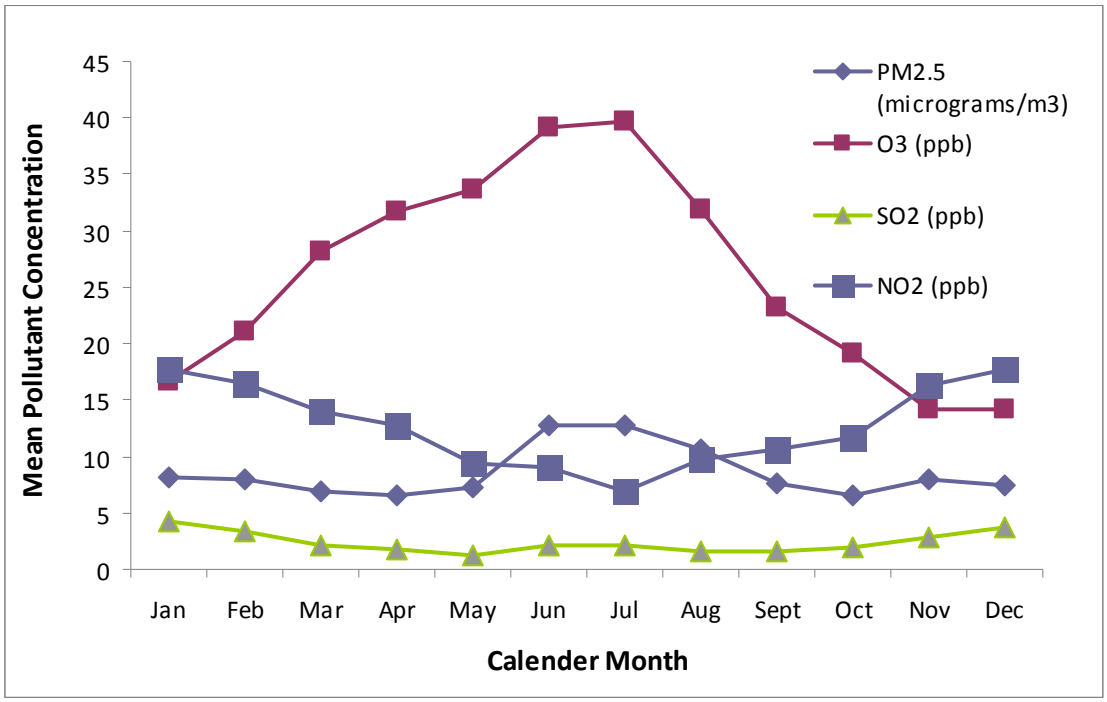


Figure 6. Mean Carbon Monoxide Concentration by Calender Month

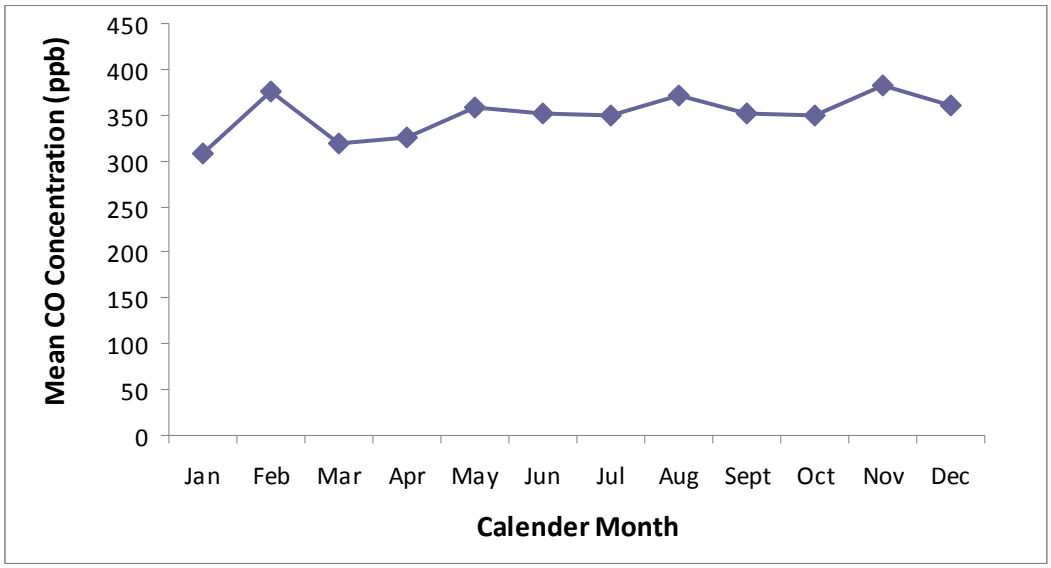


Figure 7. Mean Pollutant Concentrations by Year

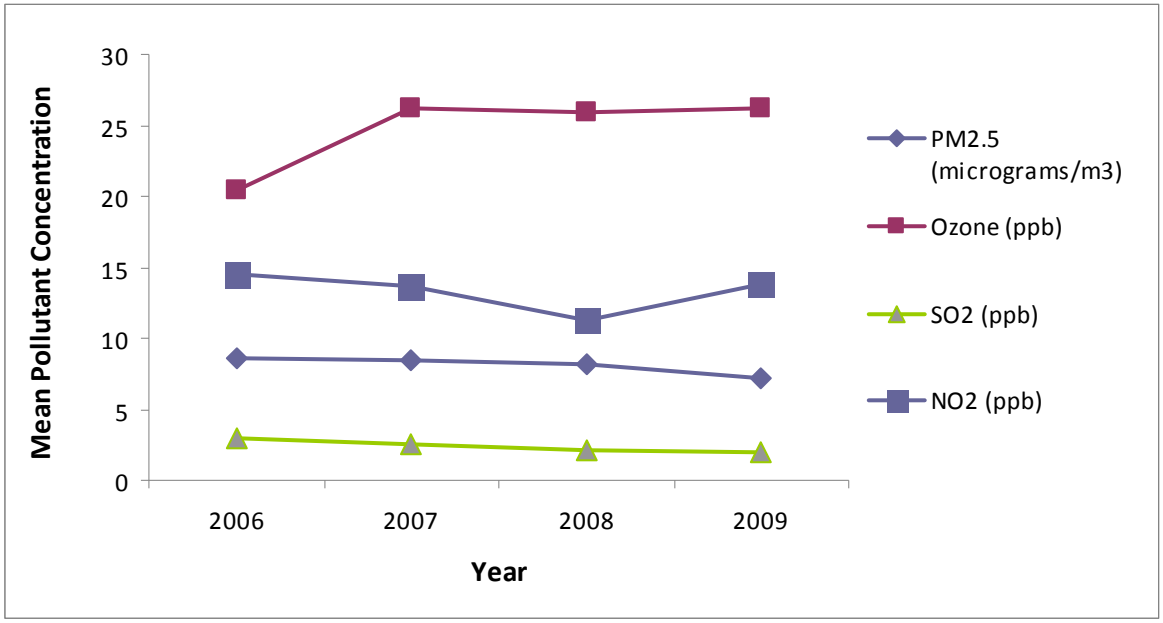


Figure 8. Mean Carbon Monoxide Concentration by Year

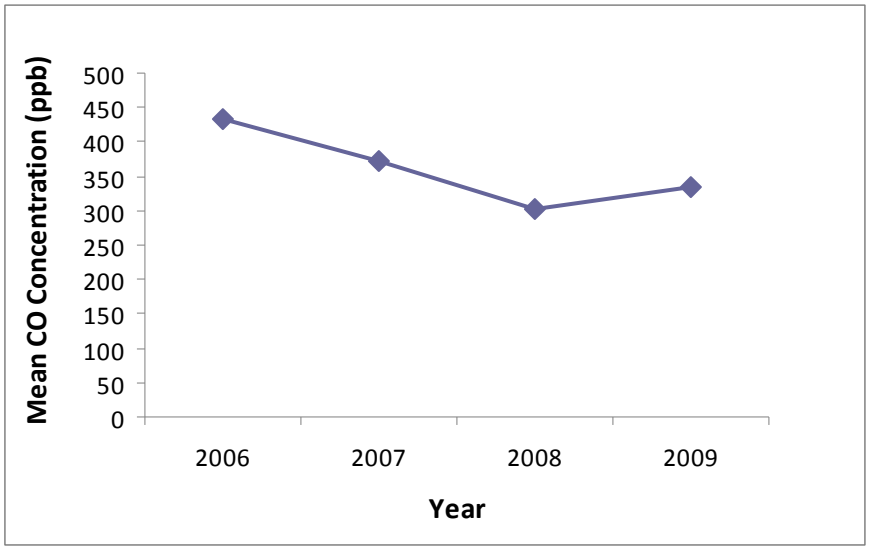


Figure 9. Percent change in plasma nitrite associated with an IQR increase in mean $PM_{2.5}$ concentration lag hours

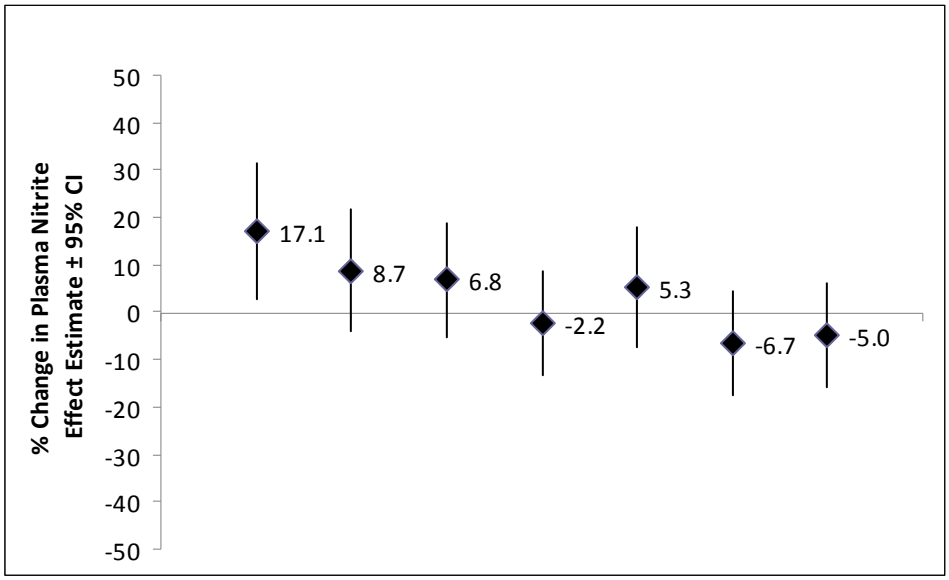


Figure 10. Percent change in plasma nitrite associated with an IQR increase in mean carbon monoxide concentration lag hours

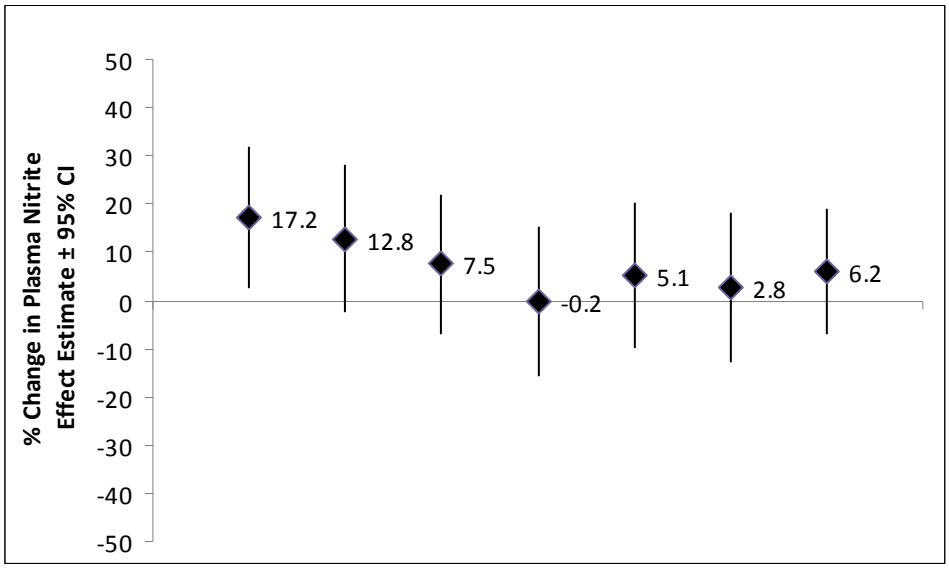


Figure 11. Percent change in plasma nitrite associated with an IQR increase in mean ozone concentration lag hours

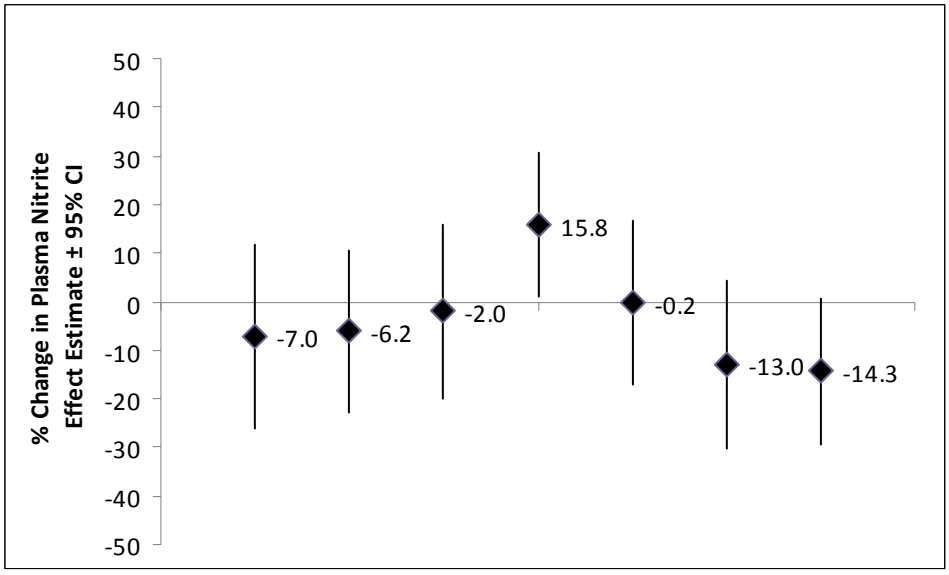


Figure 12. Percent change in plasma nitrite associated with an IQR increase in mean sulphur dioxide concentration lag hours

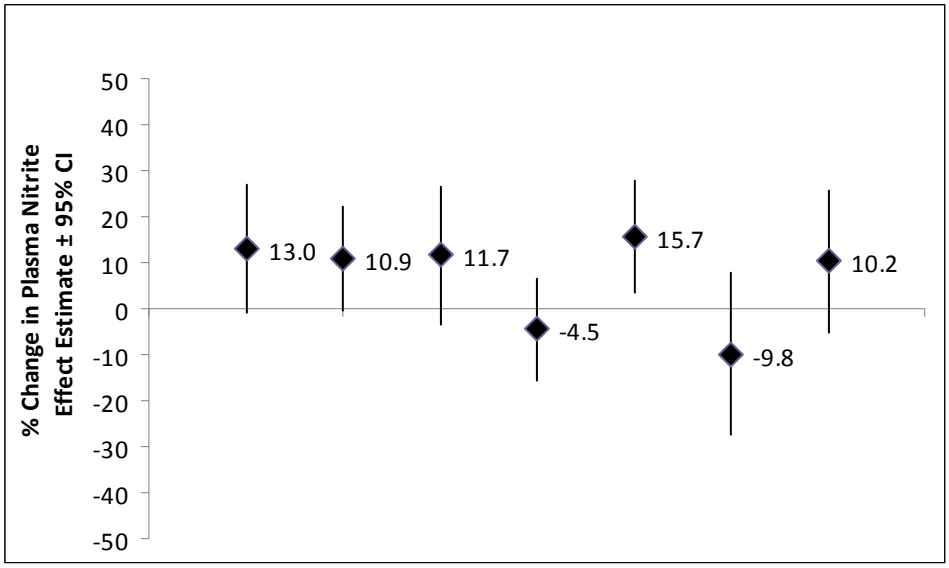


Figure 13. Percent change in plasma nitrite associated with an IQR increase in mean nitrogen dioxide concentration lag hours

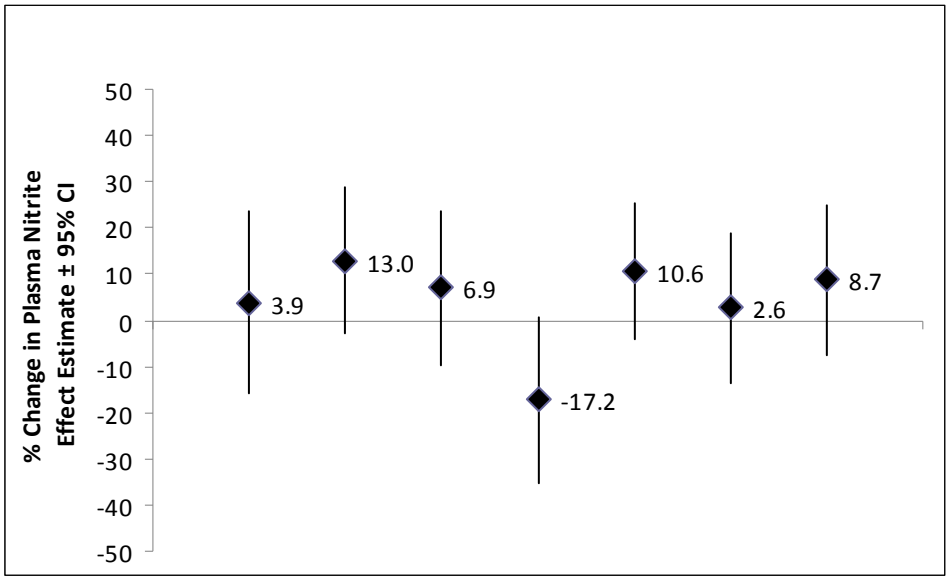


Figure 14. Percent change in PWA associated with an IQR increase in mean $PM_{2.5}$ concentration lag hours

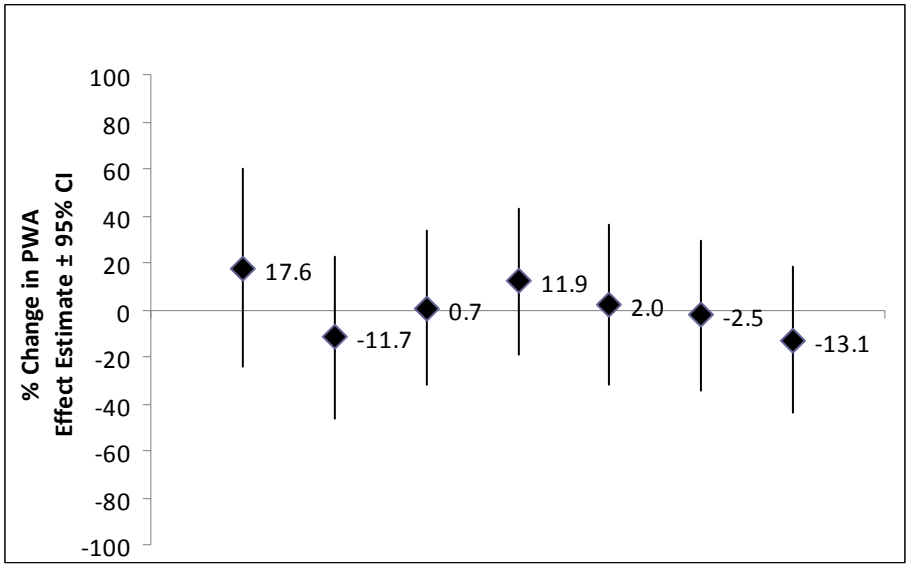


Figure 15. Percent change in PWA associated with an IQR increase in mean ozone concentration lag hours

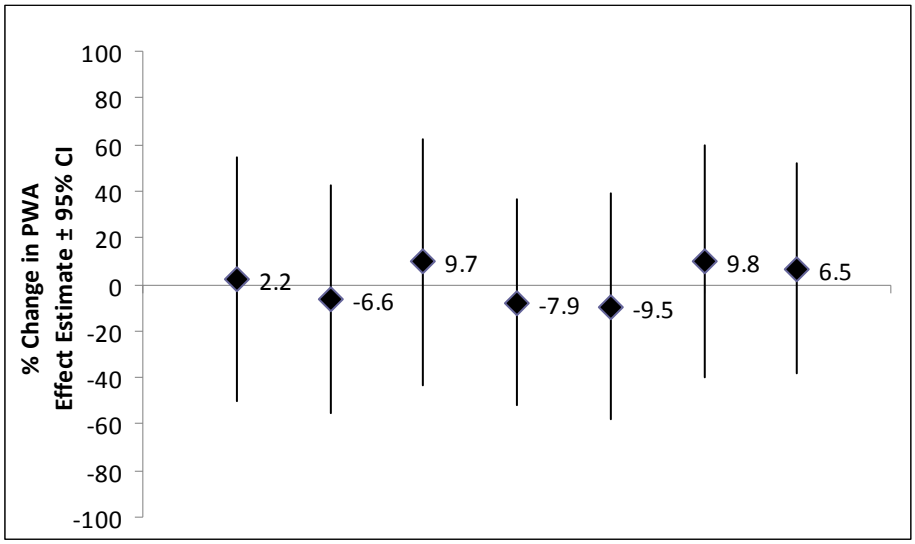


Figure 16. Percent change in PWA associated with an IQR increase in mean nitrogen dioxide

concentration lag hours

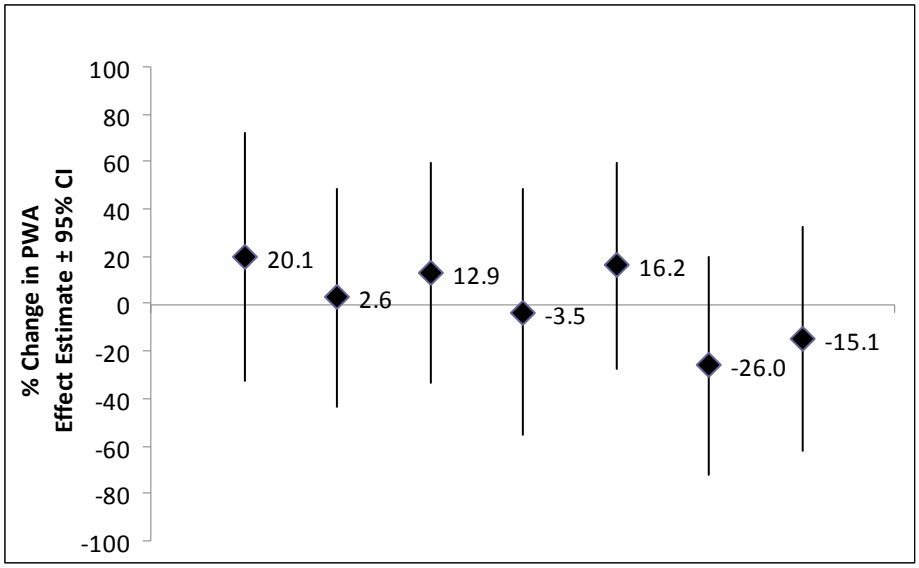


Figure 17. Percent change in PWA associated with an IQR increase in mean sulphur concentration lag hours

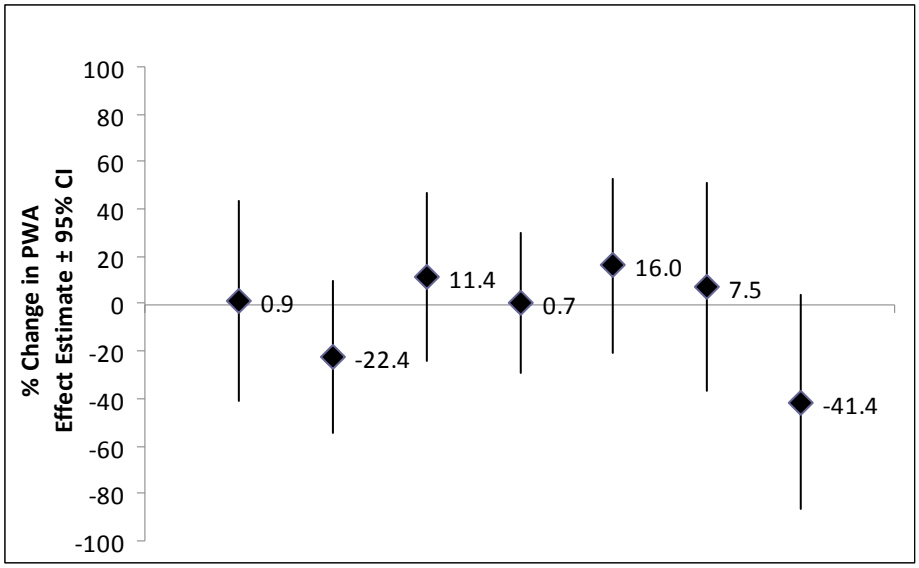


Figure 18. Percent change in PWA associated with an IQR increase in mean carbon monoxide concentration lag hours

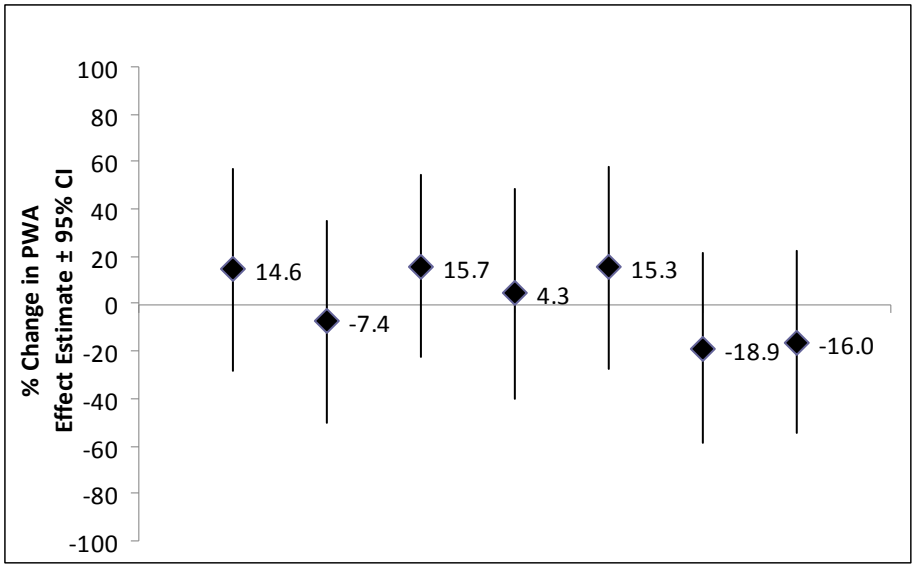


Figure 19. Percent change in PAT ratio associated with an IQR increase in mean $PM_{2.5}$ concentration lag hours

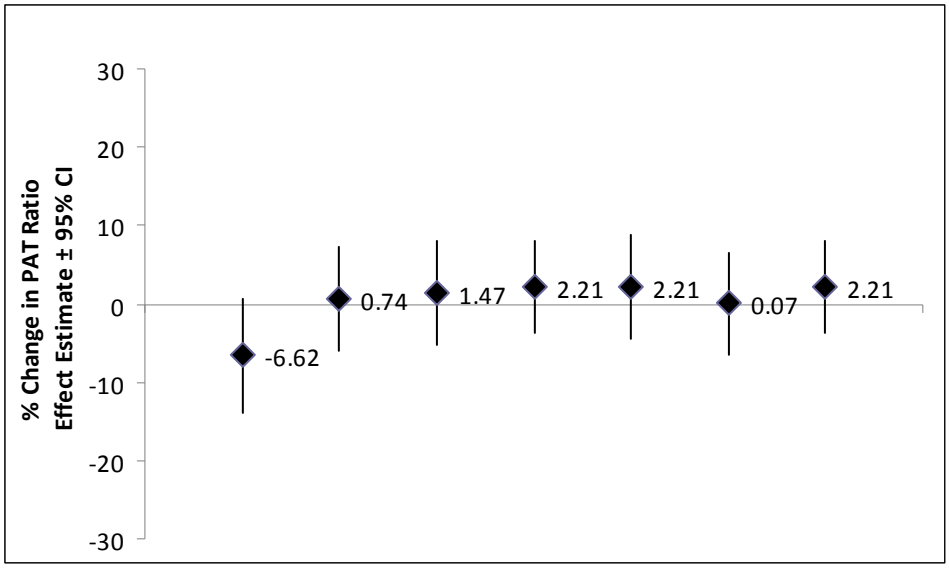


Figure 20. Percent change in PAT ratio associated with an IQR increase in mean ozone concentration lag hours

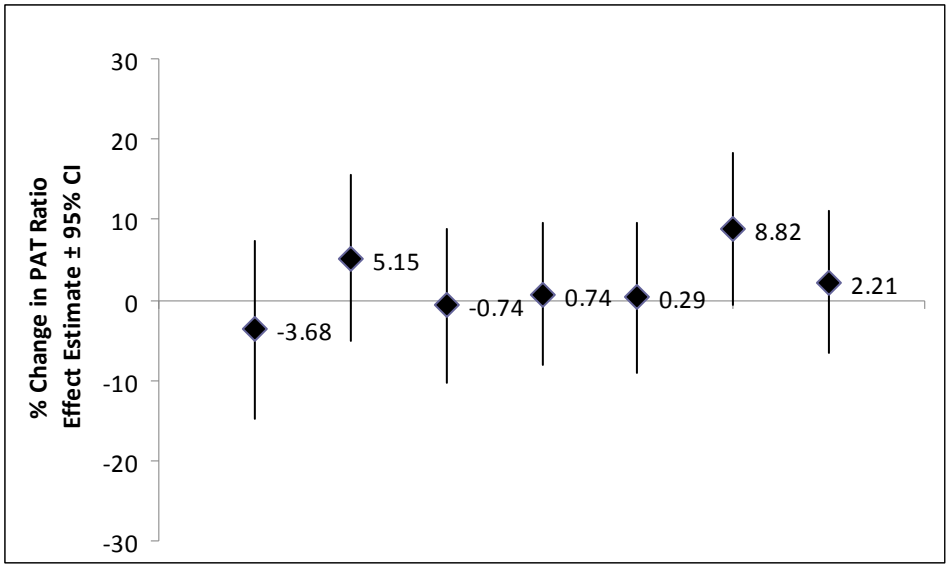


Figure 21. Percent change in PAT ratio associated with an IQR increase in mean nitrogen dioxide concentration lag hours

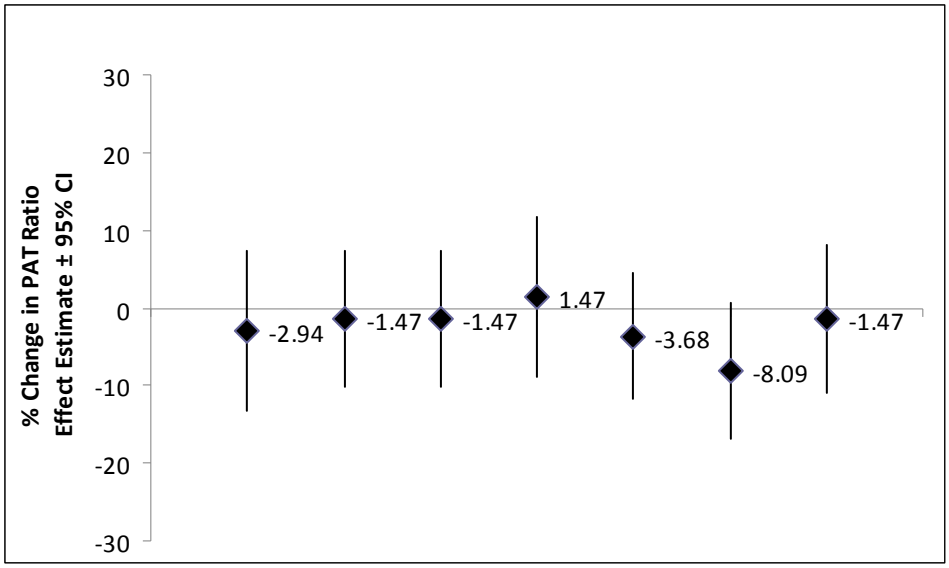


Figure 22. Percent change in PAT ratio associated with an IQR increase in mean sulphur dioxide concentration lag hours

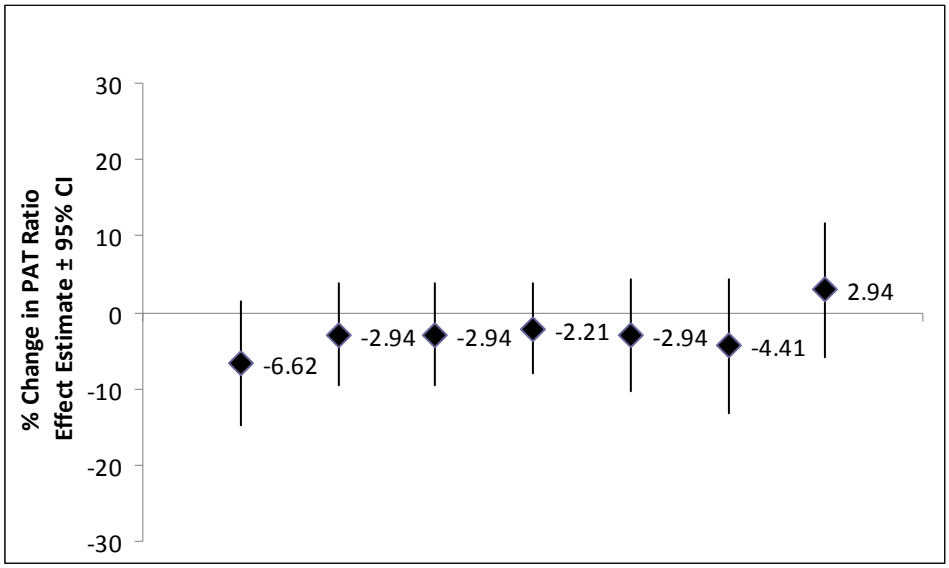


Figure 23. Percent change in PAT ratio associated with an IQR increase in mean carbon monoxide concentration lag hours

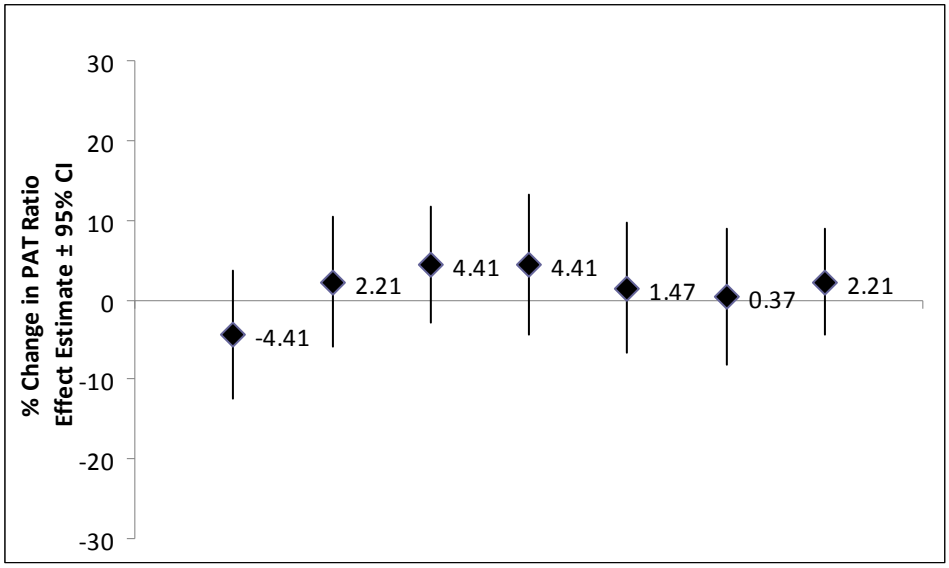


Figure 24. Percent change in Plasma Nitrite associated with an IQR increase in Mean $PM_{2.5}$ concentration lag hours including shorter time lags

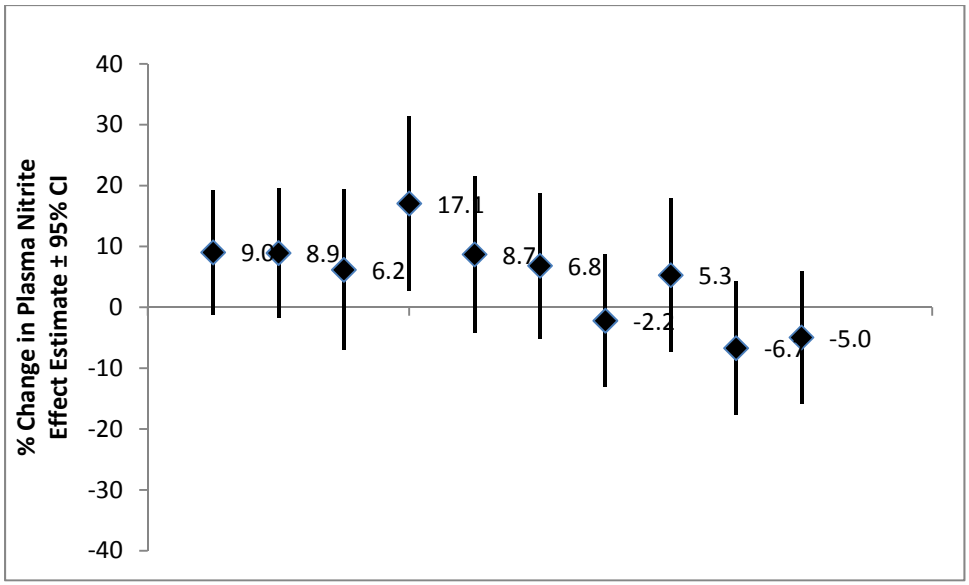
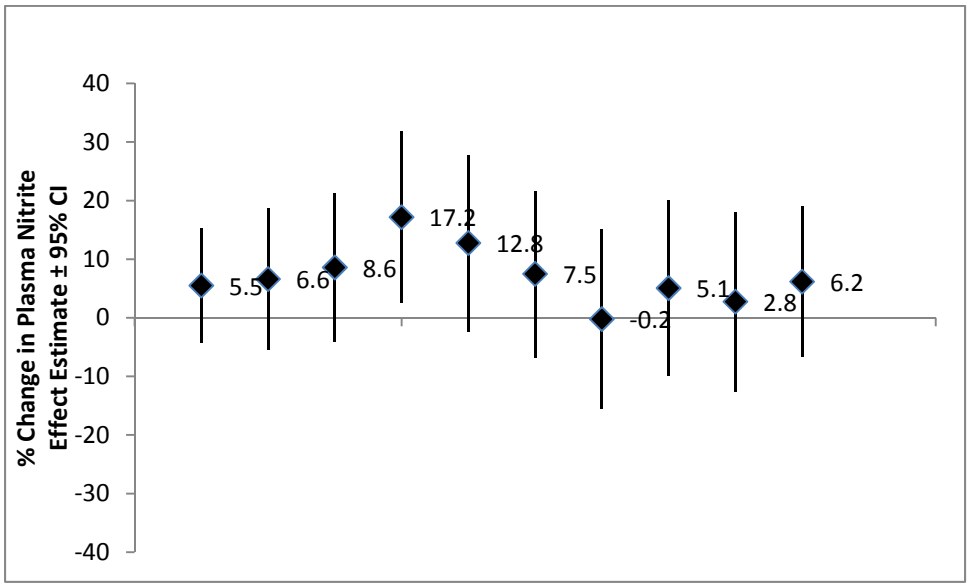


Figure 25. Percent change in Plasma Nitrite associated with an IQR increase in Mean Carbon Monoxide concentration lag hours including shorter time lags



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CONCLUSION

The research presented in this dissertation was conducted to validate the utility of the EndoPAT device in the field of environmental research. We concluded that the device does not produce reliable PAT ratio estimates when tests are repeated 2.5 hours apart on the same day and one week apart in young and healthy population. Therefore, investigators who plan to use the device repeatedly to obtain data on endothelial function should be cautious while interpreting the effects of interventions on endothelial function based on a PAT ratio alone and keep in mind that the device may not be optimal to capture the short term effects of interventions. Also, while designing studies to examine the acute effects of interventions on endothelial function using this technique in young and healthy population, investigators should be cautious regarding the use of stressful procedures and interventions as such protocols may significantly impact peripheral vascular tone as demonstrated by the changes in the pulse wave amplitude. Investigators should perhaps consider obtaining the EndoPAT data prior to performing such interventions or at the very least keep in mind that stressful protocols may have an impact on PAT measurements. Additionally, researchers should consider using only post-intervention PAT ratios instead of examining a change from baseline to post-intervention. Since this dissertation did not include examination the intra-day reproducibility of the technique for intervals longer than 2.5 hours on the same day, future studies need to be conducted to investigate if the testing can be repeated after longer time intervals such as 4 hours, 6 hours and 12 hours.

To our knowledge, this dissertation is the first study that explored the changes in pulse wave amplitude following an exposure to pollutants as well as the

reproducibility of PWA obtained from the PAT device. This is particularly important to researchers who plan to use PWA as a primary marker of peripheral vascular tone in the studies.

A decrease in the plasma nitrite concentration following a 2-hour exposure to air pollutants in a controlled environment provides a basis for larger studies to confirm the finding. Future studies are also warranted to examine the changes in plasma nitrite concentration at a post-exposure time interval longer than two hours. Future study designs also need to make every attempt to capture a blood sample following a hyperemic stimulus for measurement of plasma nitrite before as well as after hyperemic stimulus. the increase observed in the plasma nitrite concentration associated with the increase in the ambient levels of air pollutants in the first 24 hours suggested a potential role of systemic inflammation via stimulation of inducible nitric oxide synthase enzyme and generation of nitric oxide within a short period of time. To our knowledge, both these findings are novel and certainly entail further evaluation in larger panel and controlled exposure studies.

APPENDIX

A. Method of Data Collection Using the EndoPAT Tonometer Device

EndoPAT2000 Device

Peripheral arterial tonometry (PAT) is a novel method of evaluating endothelial function. This technology uses a non-invasive device called Endo-PAT tonometer 2000 (Itamar Medical, Caesarea, Israel). This device is intended for use as a diagnostic aid in the detection of coronary artery endothelial dysfunction using a reactive hyperemia procedure. It uses finger-mountable pneumatic sensors or probes specifically designed to continuously record digital arterial pulse wave amplitude (PWA). The device records beat-by-beat PWA signals and measures post-ischemic arterial responsiveness to reactive hyperemia induced by upper arm blood flow occlusion.

Data collection protocol

EndoPAT testing was conducted in a quiet, temperature and light controlled room (72 deg. F). An experienced technician who received training from the company's representative conducted these tests for all subjects. Subjects were asked to turn off cellular phones and to avoid talking. Subjects were positioned supine as shown in figure 1 below. Latex covered finger probes were applied on the tip of each index finger for measurement of PWA (Figure 1). Three readings of subject's blood pressure were recorded using a digital blood pressure monitor (Omron automatic blood pressure monitor, Model HEM-705CP). A standard blood pressure cuff was then applied on the subject's non-dominant arm for the purpose of a 5-minute occlusion of the blood flow.

This arm was referred to as the occlusion arm and the contralateral arm was referred to as the control arm. After a 5-min rest period, beat-by-beat PWA signals were collected at baseline for approximately 5 minutes following which blood pressure cuff was inflated to at least 50 mm Hg above subject's systolic blood pressure for 5 minutes. PWA signals were collected continuously during the 5-minute occlusion and for a period of at least 5 minutes following cuff deflation. Each test required approximately 30 minutes to complete. Figure 2 shows the PAT device and clinical setting requirements for PAT testing. Figures 3 and 4 show a graphical representation of PWA tracing obtained during three phases, namely, baseline, occlusion and post-occlusion from both arms and a normal hyperemic response obtained from the occlusion arm.

Automated Analysis of PWA tracings

EndoPAT software is an integral part of the EndoPAT system. It is straight-forward and easy to use. The software is used for both on-line data acquisition as well as off-line data analysis. The online display allows real-time viewing of events as they occur. The signals are recorded on the computer for subsequent review and automatic analysis. Since analysis is performed by the software, inter- or intraoperator interpretation variability is avoided. Analyzed test results are exported to an excel spreadsheet that includes multiple study parameters, calculated variables, and measures of signal quality. The mean baseline PWA is measured for the occlusion and the control arm separately and reported in standardized, arbitrary units. Then, average pulse amplitude is calculated for each 30-second intervals after cuff deflation for up to 7 minutes. Reactive hyperemia index (RHI) or PAT ratio is then automatically calculated as a ratio of the average PWA over a 1

minute period beginning 60 seconds after cuff deflation over a 3.5 minute baseline PWA. This index is commonly referred to as a Peripheral Arterial Tone (PAT) ratio, or RH-PAT index. To reduce confounding from potential systemic effects of unilateral arm ischemia, this ratio is normalized to the concurrent signal from the control arm.

Outcome variables of interest

The following variables were obtained from automatic analysis of EndoPAT data -

Mean baseline pulse wave amplitude (PWA_{occ}) Occlusion arm

(indicated by a letter B in fig 4)

Mean baseline pulse wave amplitude ($PWA_{control}$) Control arm

(indicated by a letter D in fig 4)

PAT ratio (computed using computer algorithm)

Data storage

EndoPAT studies were collected and saved automatically on a password protected computer located in room 125 of the EOHSI Clinical Center. The room was kept locked whenever not in use. Deidentified files were sent to Itamar for the purpose of quality analysis on a timely basis throughout the study period.

Quality analysis/Quality control

The main goal of the quality control process was to inspect the quality of PAT signals and perform manual correction to the automatic analysis. The scoring for each signal was determined by various parameters and divided into two different scores as follows:

Table 1. Scoring of PAT signals

Test Quality Score	**Interpretation**
1	Inadequate studies
2	Studies with some concern due to various reasons (noisy signal, inappropriate occlusion time)
3	Good quality study
Occlusion Quality score	**Interpretation**
1	Inadequate occlusion
2	Moderate to considerable breakthrough
3	Good occlusion quality

Figure 1: Finger-mountable pneumatic sensors

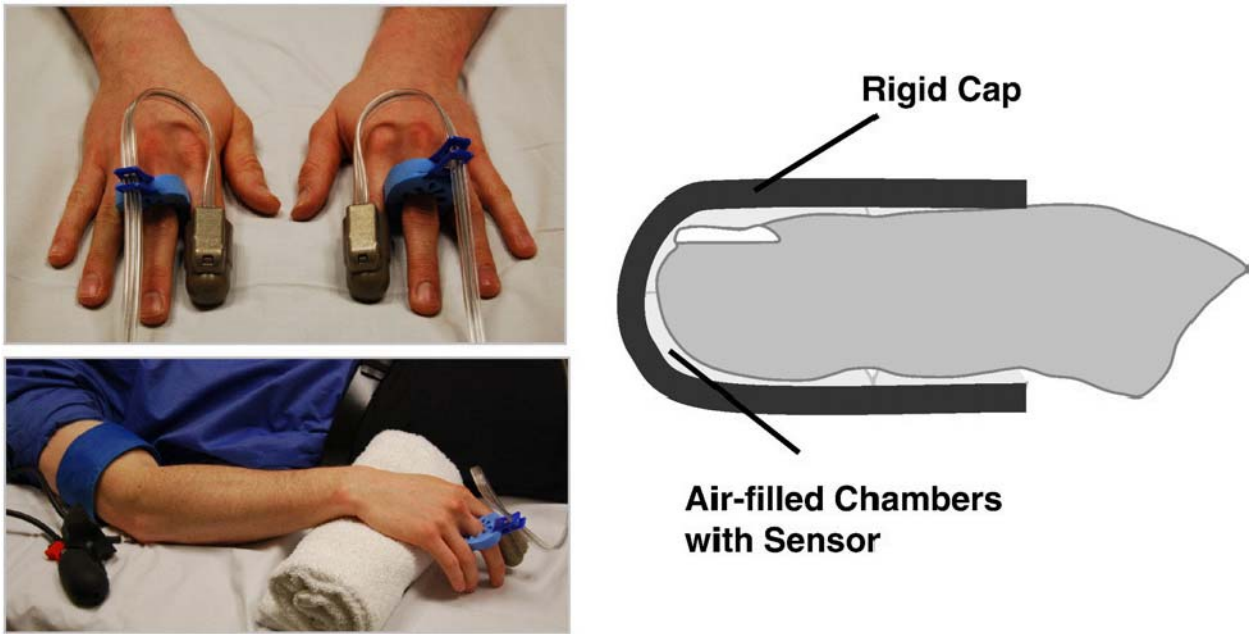
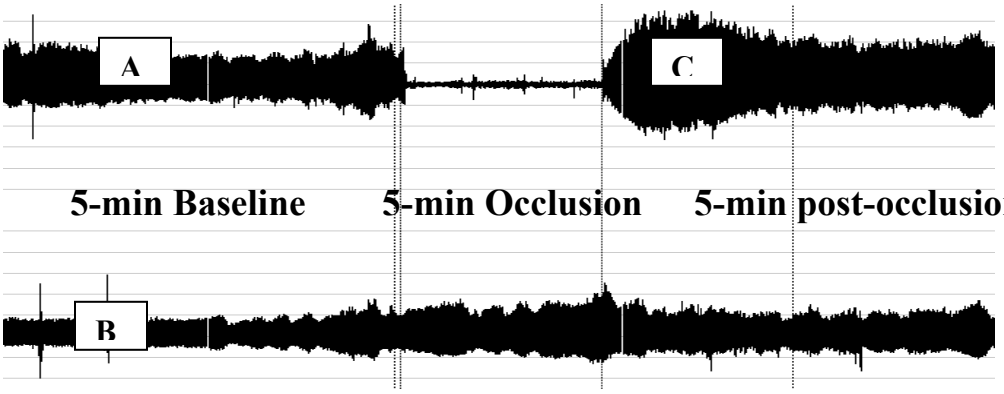


Figure 2: (Top left) The thimble-like PAT device is placed on a finger from each hand. Tubing connects the device to a recording unit that transmits data directly to a computer. (Right) The device contains air-filled chambers that are inflated to approximate diastolic pressure throughout the study. Sensors allow detection of changes in finger volume with each arterial pulsation. (Bottom left) A typical setup for a research study is shown. The hand is elevated to allow the fingers to hang freely without touching any surfaces. A cuff is placed on one forearm
that will be inflated to suprasystolic pressures to induce hyperemia.
Adapted from Hamburg N and Benjamin E. 2009.

Figure 2: Clinical settings requirement for EndoPAT tonometry procedure

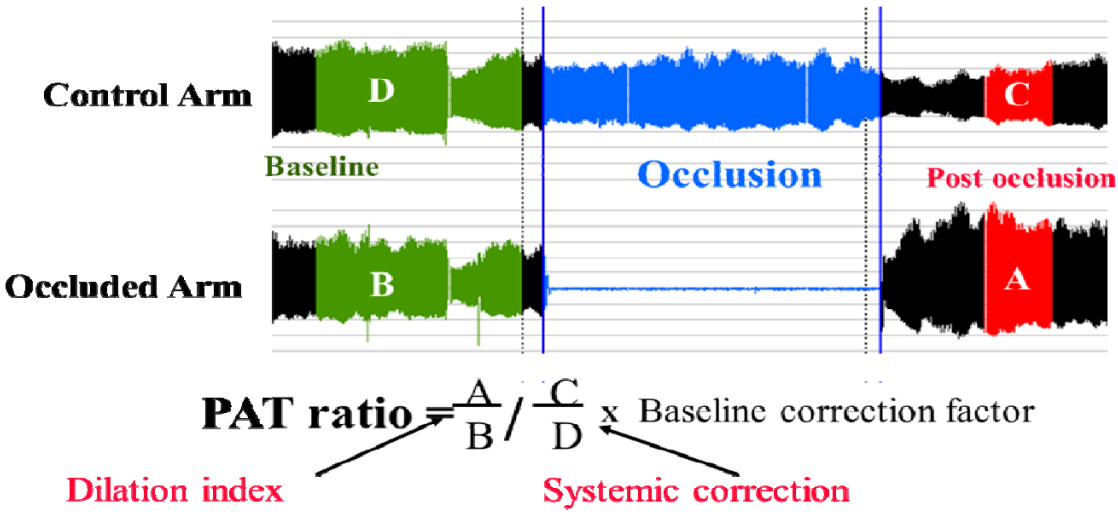
(Courtesy of Itamar-Medical Ltd, Israel)

Figure 3: Pulse wave amplitude tracing obtained from the EndoPAT tonometer



Note: A: Mean baseline pulse wave amplitude (PWA_{occ}) Occlusion arm
B: Mean baseline pulse wave amplitude ($PWA_{control}$) Control arm
C: Hyperemia response
(Courtesy of Itamar-Medical Ltd, Israel)

Figure 4: Pulse wave amplitude tracing and calculation of a PAT ratio



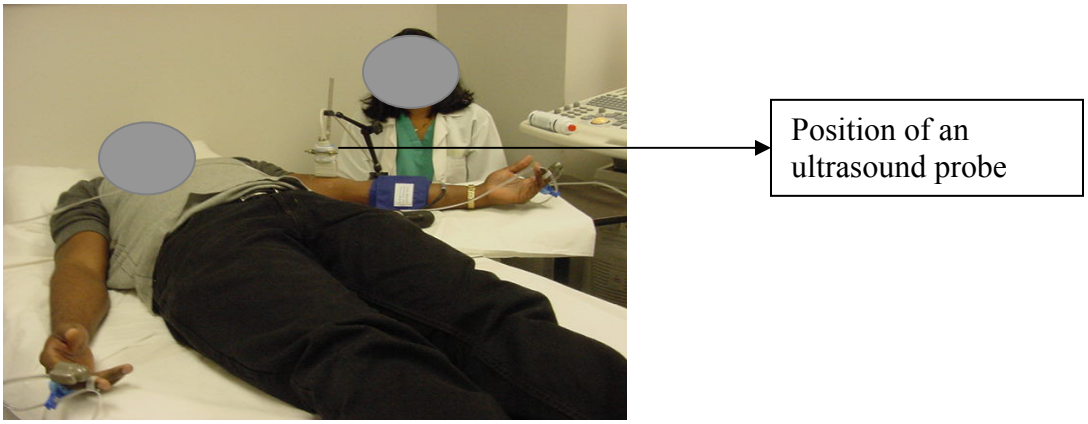
B. Method of data Collection Using Brachial Artery Ultrasound Scanning

Data collection protocol

Endothelial function measurement was performed simultaneously using two non-invasive techniques, namely the EndoPAT tonometry and brachial artery ultrasound scanning. The brachial artery ultrasound scanning was performed by a vascular technician in a quiet, temperature-controlled room (72 deg. F) with low light (Figure 5). The technician was trained by the staff at the Non-Invasive Vascular Research Laboratory at the Duke University Medical Center (DUMC) under supervision of Dr. Jason Allen to optimize data acquisition. A standardized data collection protocol as described below was used to collect brachial artery images. The vascular laboratory performed an automated analysis on brachial artery ultrasound images. The clinical settings for the ultrasound testing are as shown in figure 4. The clinical settings and room temperature were kept constant for all

studies. Prior to the testing, subjects were asked to rest in a supine position for at least 10 minutes. Ultrasound images of the brachial artery were obtained in longitudinal view, just proximal to the olecranon process of the elbow. Subjects were asked to maintain their non-dominant arm extended and slightly supinated. After the rest period, three blood pressure readings were obtained. A sphygmomanometric (or BP) cuff was placed on subject's non-dominant forearm to create a hyperemic stimulus. Electrodes were placed on subjects so that the ultrasound machine can detect heart rate throughout the test. Two baseline clips/images of the brachial artery were obtained, as well as a Doppler flow for each baseline image. The BP cuff on subject's forearm was inflated to at least 50 mm Hg above subject's systolic blood pressure for a period of 5 minutes. The time that cuff was inflated was noted. At 2 minutes after cuff inflation, a clip/image of the brachial artery and a Doppler were obtained. After 5 minutes of cuff occlusion, the cuff was deflated and a Doppler was immediately obtained. Subsequent brachial artery clips/images were obtained upon cuff deflation up to 120 seconds.

Figure 5: Clinical setting for brachial artery ultrasound scanning



Data storage

Deidenified images for each study were stored on a compact disc using a subject identification number, subjects' initials, time and date of collection of the study. The images were sent to the Non-Invasive Vascular Research Laboratory - DUMC on a timely basis for analysis.

Outcome variables of interest

- Resting or baseline brachial artery diameter (BAD) reported in millimeters
- Post-cuff deflation brachial artery diameter reported in millimeter
- Percent flow-mediated dilation (%FMD) calculated at 60 sec post-cuff deflation using the following formula –

%FMD = (Post-cuff deflation BAD – baseline BAD)/baseline BAD X 100

C. Endothelial Function Measurement Using Plasma Nitrite Assay

Venous (4.5 ml) blood samples were collected in a vacuum tube containing EDTA as an anticoagulant using standard phlebotomy procedure. These samples were collected at baseline before and immediately after exposure to DE, SOA or CA particles. Samples were immediately transported to a laboratory located in the same building on ice. A laboratory technician centrifuged these samples for 15 minutes at a maximum speed at 4 degree C and then aliquotted plasma into 1.5 ml tubes. Samples were then shock frozen in liquid nitrogen. The tubes were stored in a freezer farm at -80 degrees until they were processed further in batches. Tubes were completely thawed on ice and analyzed immediately. An experienced technician performed these analyses under the supervision of Dr. Andrew Gow. The Nitric Oxide (NO) content of plasma samples was measured by

chemical reduction-linked chemiluminescence using Sievers 280 NO analyzers (Sievers Instruments, Boulder, CO). Reduction of nitrite to nitric oxide was achieved with the use of a KI and concentrated acetic acid mixture. Nitrite concentration was reported in nanomolars (nM) and was used as an outcome variable of interest.

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