Air Pollution, Change in Retinal Vascular Caliber, and Cellular Adhesion in the Multi-Ethnic Study of Atherosclerosis (MESA)

Michael T. Young

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Reading Committee:

Joel D. Kaufman, Chair

Lianne Sheppard

Susan R. Heckbert

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Abstract

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Michael T. Young

Chair of the Supervisory Committee:
Professor Joel D. Kaufman
Departments of Environmental and Occupational Health Sciences,
Epidemiology, and Medicine

Research strongly suggests an association between acute air pollution exposure and cardiovascular events, and there is growing evidence that long-term exposure to air pollution may be associated with cardiovascular disease development. Further research on possible biologic pathways relating air pollution and cardiovascular pathology is needed to support a causal relationship and provide evidence for stricter federal air quality standards. Vascular changes and cellular adhesion processes are important pathways in cardiovascular disease, and preliminary evidence suggests they may be related to air pollution exposure. We investigated whether air pollution is associated with several markers of vascular change and cellular adhesion.

Further, we estimated whether these associations might mediate the relationship between air pollution exposure and coronary artery calcium, a clinical measure of atherosclerotic plaque.

All analyses were performed in a longitudinal cohort study, the Multi-ethnic Study of Atherosclerosis (MESA), which was designed to measure subclinical cardiovascular disease as well as cardiovascular events. We performed a longitudinal analysis of exposure to air pollutants $PM_{2.5}$ and NO_X with respect to change in vascular diameters measured in the retina (centralized retinal arteriolar equivalents or CRAE). We then performed analyses of the cross-sectional relationship between both long-term and acute exposure to air pollutants ($PM_{2.5}$ and NO_X) with fifteen different cellular adhesion proteins measured in blood or serum. Finally, we tested whether retinal vascular diameter and several adhesion proteins statistically mediate the relationship between air pollution and coronary artery calcium.

Our results indicated that greater $PM_{2.5}$ exposure was associated with more narrowing of CRAE. Comparing individuals differing by 5 $\mu g/m^3$ of $PM_{2.5}$ exposure averaged over a 7.8 year follow-up period, adjusted change in CRAE differed on average by -1.41 µm (95% CI: -3.40, 0.58, p=0.17). This estimate of -1.41 corresponds to approximately half the average change in individual CRAE values over the 7.8 years of follow-up in MESA, although this estimate of -1.41 was for a 5 $\mu g/m^3$ of $PM_{2.5}$ whereas the interquartile range of exposure for this follow-up period in MESA was 1.9 $\mu g/m^3$. We also found strong associations between greater air pollution exposure and higher levels of three specific adhesion proteins. We found that year-prior exposure to $PM_{2.5}$ and NO_X were both positively associated with the chemokine CCL21, that both pollutants had strong positive associations with the adhesion protein ICAM-1, and that $PM_{2.5}$ had a strong association with the inhibitor of matrix metalloproteinase TIMP-2. For a 5 µg/m3 interval of $PM_{2.5}$, the strengths of these associations were all roughly equivalent to 20%

of the interquartile variability observed in MESA for each respective adhesion protein. The strengths of the associations between acute $PM_{2.5}$ exposure and adhesion proteins were generally weak or null. Finally, we found evidence suggesting that CCL21 and TIMP-2 may statistically mediate the relationship between $PM_{2.5}$ and coronary artery calcium, but due to the possibility of unknown confounders between the adhesion proteins and coronary artery calcium, we cannot necessarily conclude that these proteins are causal mediators.

Overall, our results are consistent with the hypothesis that air pollution exposure may cause vascular and adhesion changes, but further research is needed to verify these results and determine whether these specific pathways could explain the relationship between air pollution and cardiovascular disease.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Chapter 1. INTRODUCTION

Air Pollution and Mortality

Exposure to ambient air pollution is ranked as the ninth leading risk factor for global mortality by the Institute of Health Metrics and Evaluation Global Burden of Disease Study (Lim et al., 2012). Ambient air pollution is a mixture of gases, including oxides of nitrogen and ozone, and particulate matter, which is typically characterized by particle size. Ambient air pollution concentrations vary globally, but even in the United States, where $PM_{2.5}$ (particulate matter less than 2.5 microns in diameter) concentration is relatively low, substantial research demonstrates risk of mortality from air pollution exposure. Estimates for the effect of exposure to long-term $PM_{2.5}$ exposure vary: a 10 $\mu g/m^3$ interval in ambient $PM_{2.5}$ exposure may be associated with a 7.3% increase in all-cause mortality (Di et al., 2017) or as much as a 76% increase in cardiovascular mortality (Miller et al., 2007). Given that exposure to ambient air pollution is widespread and often unavoidable, even relatively small risks associated with exposure result in numerous excess deaths. In the United States ambient air pollution exposure may cause 200,000 premature deaths per year (Caiazzo et al., 2013).

The association between air pollution and mortality has been replicated in many populations, but this field of research began with a series of landmark studies. The first major prospective study finding an association between ambient air pollution exposure and mortality was the Harvard Six Cities study (Dockery et al., 1993). These results were soon replicated in the American Cancer Society Cancer Prevention Study (Pope et al., 1995). The use of time-series analyses to analyze acute variations in exposure concentrations with regard to daily

hospitalization data has also been a crucial component of establishing the air pollution/mortality connection (Anderson, 2009).

Air Pollution and Cardiovascular Disease

A major focus of air pollution epidemiology research beyond mortality are the clinical morbidities and subclinical health effects of air pollution exposure. Cardiovascular disease in particular is an important effect of air pollution and a writing group of the American Heart Association has concluded that cumulative research in this area is consistent with air pollution causing not only mortality but also cardiovascular morbidity (Brook et al., 2010). The association between air pollution exposure and blood pressure is well established (Brook and Rajagopalan, 2009). Emerging research suggests that air pollution may be associated with direct measures of atherosclerosis such as intima media thickness, an indicator for vessel wall thickening (Bauer et al., 2010; Künzli et al., 2005; Perez et al., 2015), and with vessel calcification (Hoffmann et al., 2007; Kälsch et al., 2014; Kaufman et al., 2016). Nevertheless, better insight into the biological mechanisms of air pollution research will inform relevant types of pollutants, exposure level, and susceptible populations. Furthermore, air pollution research is an extrinsic, modifiable risk factor for cardiovascular disease and therefore can provide unique insight generally into cardiovascular disease pathobiology. For these reasons, researchers on the relationship between cardiovascular disease and air pollution exposure have taken a particular interest in the biological mechanisms for this association.

Air Pollution: Biological Mechanisms for Cardiovascular Disease

While there is evidence consistent with a causal effect of air pollution on cardiovascular disease, the biological pathways for this relationship are unknown. Three general models for the pathobiologic response to air pollution exposure have been proposed (Brook et al., 2010): Air pollutants may act directly on lung tissue leading to a cascade of inflammatory cytokines, particles may cross through the lungs into the bloodstream leading to direct effects of particulate matter on systemic circulation, and particles may act on pulmonary neuroreceptors, leading to systemic autonomic nervous system activation.

Air pollution mechanisms research relies on the major contributions of experimental exposure in humans and animal models. Human controlled exposure research strongly suggests that air pollution causes acute vascular changes. Controlled diesel exhaust exposure causes vasoconstriction (Peretz et al., 2008), ST-segment depression (Mills et al., 2007), and blood pressure increases (Cosselman et al., 2012). Recent evidence indicates that these blood pressure changes may be mediated by adrenergic receptors (Cosselman et al., 2016) suggesting air pollution may alter blood pressure directly through autonomic changes. Alteration of vessel tone could potentially explain the relationship between air pollution exposure and cardiovascular disease events. Increased blood pressure can contribute to atherosclerotic lesions and permanently narrowed vessels can increase the likelihood of a vessel occlusion.

If air pollution causes vascular and blood-pressure related changes, these changes likely involve the endothelium. The endothelium is the set of cells that line the inside of vessels, including resistance arteriolar resistance vessels which regulate blood pressure. Endothelial cells are indirectly involved in regulating vessel tone through production of local vasodilators and vasoconstrictors which in turn act on smooth muscle cells. The most important local agent

produced in the endothelial cells is nitric oxide, a potent vasodilator. Endothelial cells play a central role in flow-mediated dilation and reactive hyperemia, and also mediate the effect of the autonomic nervous system on vessel tone (Amiya et al., 2014). If air pollution causes a sympathetic response which alters blood pressure, then these effects may be mediated by the endothelium.

Furthermore, endothelial control of vessel tone and therefore blood pressure is highly sensitive to reactive oxygen species (Touyz, 2004). Air pollution itself may directly deliver reactive oxygen species to the vascular endothelium or indirectly through pulmonary-derived factors (Mills et al., 2009). Increased reactive oxygen species may disrupt nitric oxide-dependent vasodilation, thereby causing endothelial changes which could affect total peripheral resistance and therefore blood pressure (Craige et al., 2015).

Microvasculature and Retinal Measurement

Control of blood pressure occurs primarily through the changes in vascular tone in the small vessels or the microvasculature. The microvascular arterioles are the primary resistance vessels in the body that are involved in regulating blood pressure through a complex set of pathways involving the endothelium and autonomic nervous system (Segal, 2005). If air pollution permanently alters blood pressure or vascular function, these effects may be detectable as changes in vessel tone. However, characterizing the microvasculature non-invasively in human subjects requires creative measurement techniques. Retinal photography is one such non-invasive method for characterizing microvascular diameter and shape. Retinal arteriolar and venular diameters are also independently associated with cardiovascular risk factors such as hypertension, diabetes, obesity, and dyslipidemia (Wong et al., 2006a), suggesting this

measurement may reflect underlying pathological changes that are universal to a variety of disease processes. Cross-sectional analyses have shown that retinal arteriolar and venular microvasculature diameters vary with acute (Louwies et al., 2013) and chronic air pollution exposure (Adar et al., 2010). But to our knowledge no study has investigated the longitudinal association between chronic air pollution exposure and measures of retinal microvasculature.

Adhesion Processes

While a large body of evidence suggests that air pollution may be involved in alteration of vascular function and endothelial behavior, additional research also suggests that air pollution may have inflammatory effects. Animal models and human controlled exposures demonstrate air pollution induces pulmonary-derived inflammatory factors such as cytokines which are released into systemic circulation (Cosselman et al., 2015; Mills et al., 2009).

At the intersection between these two mechanisms of cardiovascular disease—vessel tone and inflammation—is the process of cellular adhesion. Cellular adhesion describes the interaction or attachment between cellular surfaces and other cells or substrates. Alteration of adhesion processes plays an important role in cardiovascular disease development (Blankenberg et al., 2003). Cellular adhesion occurs through direct cell-cell rolling and adhesion, extracellular signaling, alteration of the extracellular matrix, and regulation of cellular proliferation. It is through this process that immune cells attach themselves and infiltrate the endothelium in the process of plaque formation. Although there is a large amount of epidemiological evidence linking air pollution exposure and cardiovascular disease, the extent to which air pollution affects adhesion processes is largely unknown.

Summary of Chapters

The following analyses were chosen to better characterize the acute and long-term effects of air pollution on both the microvasculature and adhesion processes in the context of a single observational study. This dissertation was undertaken in order to elucidate whether these specific pathways play a central role in the effect of air pollution on cardiovascular disease.

In Chapter 2, the objective of was to (A) identify associations between long-term air pollutant exposure ($PM_{2.5}$, NO_X) and longitudinal changes in retinal arteriolar and venular microvascular diameters and (B) examine the cross-sectional association between acute $PM_{2.5}$ exposure and retinal microvascular diameters.

In Chapter 3, the objective was to estimate the associations of chronic and acute air pollution concentrations with soluble protein concentrations for a suite of adhesion-associated proteins involved in atherosclerotic vascular damage including various specific adhesion proteins, selectins, cytokines, growth factors, and metalloproteinases measured in serum and plasma.

In Chapter 4, we aimed to determine whether findings from Chapters 2 and 3 might explain the association between air pollution and atherosclerosis as measured by the clinical outcome coronary artery calcium. We performed a statistical mediation analysis on one retinal marker and three adhesion proteins with respect to the relationship between $PM_{2.5}$ and NO_X exposure and longitudinal change in coronary calcification.

Finally, in chapter 5 we summarize our findings in the context of the literature and make recommendations for future research.

Chapter 2. AIR POLLUTION EXPOSURE AND RETINAL VESSEL DIAMETER

2.1 INTRODUCTION

Air pollution exposure has been identified as a risk factor for cardiovascular disease, but the biologic mechanisms for this relationship are not well understood. One of several proposed pathways involves alteration of vascular function (Krishnan et al., 2012a; Pope and Dockery, 2006). Controlled exposure studies show that particulate matter inhalation is associated with measures of large artery reactivity including brachial artery diameter (Brook et al., 2002), but limited research exists on the effect of air pollution exposure and changes in the small vessels or microvasculature.

Retinal photography is a noninvasive method for characterizing microvascular diameter and shape. Retinal arteriolar and venular diameters are associated with cardiovascular risk factors such as hypertension, diabetes, obesity, and dyslipidemia (Wong et al., 2006a). Additionally, abnormalities in the retinal microvasculature predict cardiovascular events independent of traditional risk factors (Wong et al., 2003). Cross-sectional analyses have shown that retinal arteriolar and venular microvasculature diameters vary with acute (Louwies et al., 2013) and chronic air pollution exposure (Adar et al., 2010). Smoking has been associated with longitudinal changes in retinal vessel diameters (Kifley et al., 2007). However, to our knowledge no study has investigated the longitudinal association between chronic air pollution exposure and measures of retinal microvasculature. The objective of this study was to (A) identify associations between long-term air pollutant exposure ($PM_{2.5}$, NO_X) and longitudinal changes in

retinal arteriolar and venular microvascular diameters and (B) examine the cross-sectional association between acute $PM_{2.5}$ exposure and retinal microvascular diameters.

2.2 METHODS

Study Population

The Multi-Ethnic Study of Atherosclerosis (MESA) is a national multi-site cohort study designed to longitudinally collect subclinical and clinical measures of atherosclerosis in a diverse population. MESA participants (n= 6,814) were recruited from July 2000 to August 2002 from six metropolitan areas in the United States (Los Angeles, California; Minneapolis-St. Paul ("Twin Cities"), Minnesota; Chicago, Illinois; Baltimore, Maryland; Winston-Salem, North Carolina; and New York, New York). Eligible individuals identified as Caucasian, Chinese, African-American, or Hispanic. At recruitment, participants were 45 to 84 years old and were free of clinical cardiovascular disease. Participants received five medical examinations over the course of approximately ten years of follow-up. At each examination, MESA staff measured cardiovascular risk factors including anthropometry, blood pressure, and circulating biomarkers and collected information on participant demographics and risk behaviors.

Retinal Measures

Central retinal arteriolar equivalents (CRAE) and central retinal venular equivalents (CRVE) are the two primary outcomes for this analysis. These measures correspond to the average diameters of the largest six arterioles and venules respectively in a defined area surrounding the optic disk (Knudtson et al., 2003). A computer-assisted image analysis approach

for calculation of CRAE and CRVE has been previously validated using repeated photographs in the same individual and showed reliability coefficients for images taken two weeks apart of 0.74 for CRAE and 0.79 for CRVE (Couper et al., 2002). These reliability coefficients were not appreciably different than those for a similar validity analysis of blood pressure in the same cohort.

Participants underwent retinal photography at the second and fifth MESA examinations. Therefore, these two examinations constitute baseline and follow-up respectively for the longitudinal analysis of CRAE and CRVE. Follow-up between these examinations was approximately 7.8 years. Retinal photographs were taken using a 6.3 megapixel digital nonmydriatic camera and CRAE/CRVE were calculated at a centralized research lab by trained graders using the IVAN (Interactive Vessel Analyzer) software (University of Wisconsin)(Wong et al., 2006a). Diameters of all arterioles and venules were measured at fixed distances from the optic disc margin and averaged using formulas for central retinal venular equivalents (CRVE) and central retinal arteriolar equivalents (CRAE) (Wong et al., 2006a). These published measures of vessel diameters are independent of image scale and are robust to variance in the number of measured vessels (Knudtson et al., 2003). For the present analysis, we used CRAE/CRVE calculated from the right eye unless the value was missing for that eye in which case the left eye was used.

Exposure Assessment

To estimate acute and chronic effects of air pollution on the retinal microvasculature, we estimated participant exposures to $PM_{2.5}$ on two separate time-scales: daily ("acute") and multi-year periods ("long-term"). Due to the highly variable spatial characteristics of NO_X and lacking

sufficient spatial NO_X monitoring coverage on the acute time-scale, we only estimated the effects of long-term NO_X exposure.

Long-term Exposure Assessment

Long-term estimates of $PM_{2.5}$ and NO_X exposure, representing multi-year exposures, were calculated separately using validated MESA-specific spatiotemporal statistical models for predicting participant air pollution concentrations at their home address during the study period (Keller et al., 2015). Briefly, these exposure models utilize smooth spatial and temporal trends and incorporate meteorological information, roadway dispersion modeling, and land-use characteristics such as distance from roadway to characterize fine-scale traffic-related air pollution. We averaged participant-specific estimates over the period from the time of the baseline retinal image taken at examination 2 to the follow-up image taken at examination 5. We also averaged participant-specific estimates over the year prior to examination 2 (this was used as a baseline adjustment to account for pre-baseline exposure). These long-term estimates of $PM_{2.5}$ and NO_X exposure were produced from separate models.

Acute Exposure Assessment

In order to obtain daily estimates of participant exposure to $PM_{2.5}$, we used a single centrally-located EPA monitoring station for each of the six study sites as an estimate for city-wide average $PM_{2.5}$ concentrations. Using a single monitor for a city is a previously utilized approach to acute exposure assessment (Adar et al., 2010) and is appropriate for this pollutant because daily variations in $PM_{2.5}$ are relatively spatially homogenous. Since spatial trends in $PM_{2.5}$ are well-characterized by our long-term exposure model, these short-term city-wide

pollution levels provide complementary temporal variation in order to estimate associations between acute air pollution exposure and the health outcomes. Exposure contrast for the acute analysis is temporal (rather than spatial), leveraging differences in the days that individual participant examinations occurred.

To eliminate the possibility of confounding by temporal factors, the complete daily time-series of monitoring data was pre-adjusted for meteorologic factors and time (Szpiro et al., 2014). This approach to confounding adjustment is preferable to direct adjustment in the health model because pre-adjustment uses the complete time-series of monitoring data, including days on which MESA examinations did not occur, thus allowing for more precise estimation of the effect of temporal factors on daily $PM_{2.5}$ variation. We used polynomial splines with pre-specified degrees of freedom to model the time-series of $PM_{2.5}$ monitoring data from a single AQS monitor in each city. Separate models were fit for each city. Variables included were temperature (6 degrees of freedom per year), relative humidity (6 degrees of freedom per year), and time (12 degrees of freedom per year).

The exposure estimates for the acute effects analysis were these preadjusted city-specific monitoring values from days lagged relative to the examination date of each participant. We considered the following averaging periods: the day of the examination, the day prior to the examination (lag 1), and periods corresponding to the averages of lag days 1 and 2, lags 1 through 3, lags 1 through 4, and lags 1 through 5. Estimation of daily exposure estimates of $PM_{2.5}$ did not incorporate the MESA spatiotemportal model or MESA monitoring data due to limitations of the MESA monitoring data (fixed two week intervals are the shortest resolvable averaging period for estimates produced by the MESA-specific models).

The acute $PM_{2.5}$ exposures we employed are mean-centered for each study site as a result of pre-adjustment. This city-specific pre-adjustment approach yields residuals devoid of city-specific mean values. However, we choose to adjust for study site due to concerns regarding confounding by region, so city-specific differences in exposure contrasts are not relevant and their absence does not affect the estimation of acute $PM_{2.5}$ exposure on retinal measures.

Statistical models

We used a mixed effects model to estimate rate of change in the retinal measures over the study design. This approach includes a modeled baseline value (in this case, examination 2 retinal measures) rather than an adjustment for the baseline value to avoid introducing error into the progression estimate (Gassett, 2013; Gassett et al., 2015). The mixed model using the following equation for subject i and visit index v:

$$Y_{iv} = [\alpha_0 + X_{i0}\alpha_1 + a_i] + [t_{iv}\beta_0 + W_{iv}t_{iv}\beta_1 + t_{iv}b_i] + [U_{iv}\gamma_1 + \epsilon_{iv}]$$

where Y_{iv} is the retinal measurement for subject i at follow-up visit v (visits 2 and 5), ϵ_{iv} are the residuals (the error structure allowed a random intercept for participant which was separate but assumed to be uncorrelated with observation-specific error), t_{iv} is the time from examination 2 to examination 5 for subject i, X_{i0} are time-invariant cross-sectional variables at baseline (examination 2) for subject i, W_{iv} are variables for which a progression effect is estimated, and U_{iv} are time-varying variables to adjust measurements at visit v for subject i for variables that could change between examinations.

Multi-year Exposure assessment

The long-term average individual estimates of the air pollution exposure over examinations 2 to 5 is a variable in the matrix W_{iv} and therefore the vector β_1 contains the parameter of interest for the longitudinal analysis, corresponding to an estimated rate of change in the outcome statistically attributable to the exposure over the follow-up period. The effects of each pollutant ($PM_{2.5}$ and NO_X) were estimated independently in separate models.

Acute Exposure Assessment

We estimated acute effects using a repeated measures style approach (i.e. cross-sectional effects over multiple time points) using the same mixed effects model without adjustment for long-term exposures. This allowed us to adjust for progression due to covariates as precision or confounding variables as well as adjustment for time-varying effects. For these models, the preadjusted acute exposure was included in the model as variable in the matrix U_{iv} (i.e. modeled as a time-varying exposure) and therefore the parameter of interest in this analysis is an element of γ_1.

Exposure Intervals

The longitudinal analysis effect estimates correspond to rates per units of a pollutant, so we chose scaling factors for these effects according to 1) a relevant exposure interval and 2) an amount of follow-up time. The exposure interval for $PM_{2.5}$ was chosen to be 5 $\mu g/m^3$ for consistency with other literature. The exposure interval for NO_X was chosen to be 29.2 ppb which corresponds to the MESA interquartile range of average NO_X exposure over the examination 2 through 5 follow-up period. The amount of follow-up time used to scale the effect

estimates was 7.8 years, which is the average follow-up time from examinations 2 to 5. Therefore, the effect estimate for long-term $PM_{2.5}$ exposure represents a change over time in the retinal measure comparing individuals differing by 5 $\mu g/m^3$ in long-term $PM_{2.5}$ exposure per 7.8 years. For long-term NO_X exposure, the effect estimate represents a change over time in the retinal measure comparing individuals differing by 29.2 ppb in long-term NO_X exposure per 7.8 years. Although the statistical model (and the limitation of having only time points) necessitates estimation of an effect representing a constant rate of change (in units of μm/yr) statistically attributable to air pollution exposure, we recognize that the slope is not necessarily constant over this period. In presenting the results, we represent the rate as the average estimated change over the average length of the follow-up period (by rescaling the effect estimates to units of 7.8 years) to emphasize that this estimate does not necessarily represent a constant linear amount of change each year and also to represent the total size of the effect observed over follow-up.

The interval size of 5 $\mu g/m^3$ of $PM_{2.5}$ exposure was used for the longitudinal analysis for consistency with other research, although the interquartile range corresponding to average $PM_{2.5}$ exposure over the period from examinations 2 to 5 was only 1.9 $\mu g/m^3$. The results for the acute analysis were also scaled to represent a difference of 5 $\mu g/m^3$ cross-sectionally (and this value is close to the acute interquartile ranges are presented in Table 2.4).

City-Specific Effects

To assess how model estimates differed by study site and whether overall effects might be dominated by a single study site, we fit longitudinal models separately for each city. These are stratified models rather than statistical interactions since our interest in this analysis was purely

diagnostic rather than from an *a priori* interest in effect measure modification. We compared city-specific results to the overall effects in order to assess the influence of individual study sites.

Interaction by Age, Gender, and Anti-hypertensives

We performed exploratory analyses for effect modification by age, gender, and classes of antihypertensive drugs. Our particular interest in antihypertensive drugs was to determine whether direction of effect by classes of antihypertensive might suggest possible biologic pathways or disease etiologies. To avoid confounding the interactions by hypertensive status, the anti-hypertensive interaction models were restricted to subjects with hypertension. Due to the exploratory nature of this analysis, we did not formally test for statistical interaction.

Confounding adjustment

We selected potential confounders *a priori* and used a staged approach to present adjusted effect estimates. The *minimally adjusted* model included baseline (examination 2) terms (X_{i0}) and progression from baseline terms (W_{0v}) for age, gender, race, education, site, and the long-term estimate of the pollutant averaged over the year prior to the participant's examination 2. The *fully-adjusted* model includes variables from the minimally adjusted model plus time-varying terms (U_{iv}) and progression from baseline terms (W_{0v}) for the following variables: smoking status (former, current, never), BMI (continuous), diastolic blood pressure (continuous), systolic blood pressure (continuous), diabetes status (normal, impaired, untreated diabetes, treated diabetes), weekly intentional exercise (hours), anti-hypertensive medications (yes/no), and lipid-lowering medications (yes/no). The adjustment approach was the same for the acute models, except the long-term pollution in the year prior to examination 2 was not included in the

models for the acute effects analysis. The acute effects model also did not adjust for the long-term exposure between examinations 2 and 5, nor did the long-term exposure model adjust for acute effects.

For the effect modification analysis (long-term effects only), we used an altered fully adjusted models that only included progression terms and did not include time-varying terms for each variable.

2.3 RESULTS

Participant Characteristics

MESA participants at recruitment were white (38%), Chinese (12%), black (28%), or Hispanic (22%). Approximately 53% of participants were women. The mean age at examination 2 was 64 and the mean BMI was 28 kg/m^2. Participants were 46% smokers, 43% former smokers, and 11% current smokers. Average follow-up was 7.8 years (interquartile range: 7.6-7.9 years, standard deviation=0.37).

Missingness

Retinal photographs were taken at examinations 2 and 5, so the analyzed cohort differed from the MESA cohort at recruitment (examination 1). There were 581 subjects who did not participate in examination 2 (6814 versus 6233) and there were an additional 1,618 subjects who did not participate in examination 5 (6233 versus 4615) (Table 2.2). Additional missingness occurred due to incomplete or unreadable retinal photographs (4615 with any follow-up at examinations 2 and 5 compared to 3988 with measured CRAE and 4031 with measured CRVE). For the purpose of the mixed effects model participants were not required to have a follow-up

retinal measurement to contribute to estimation of baseline effects in the model, but only those with two measurements contributed to progression estimates.

Examination 2 characteristics of MESA participants with retinal measures who attended examinations 2 and 5 were similar to the MESA cohort at examination 2 with retinal measures. There was a slight trend for more educated individuals to stay in the cohort at examination 5 (35% college or graduate educated with retinal measures at examination 2 cohort compared to 39% college or graduate educated with retinal measures at examinations 2 and 5) (Table 2.1). Average participant age at examination 2 was 64 years in the full examination 2 cohort compared to 62 years in those with retinal measures at examinations 2 and 5. Additional missingness occurred due to incomplete or unreadable retinal photographs, although this is unlikely to be related to exposure or outcome, and missingness in exposure predictions, although these sources of missingness were smaller than missingness from loss to follow-up (Table 2.2).

Retinal Measures

The average follow-up between retinal examinations was 7.8 years. Mean CRAE was 144 µm (interquartile range 135-153) at examination 2 and 141 µm (132-151) at examination 5. Mean CRVE was 214 µm (interquartile range199-228) at examination 2 and 206 µm (191-221) at examination 5. Individual differences between follow-up and baseline measurements are presented in Table 2.3. CRAE decreased by about 3 µm per 7.8 years on average and CRVE diameter decreased by about 7.6 µm per 7.8 years on average. Notably the 75^{th} percentile for both measures was positive (3.8 µm/yr for CRAE and 1.3 µm/yr for CRVE) indicating that for over a quarter of individuals retinal diameters measures increased (Table 2.3). Change in CRAE and CRVE were positively correlated (Pearson r=0.33) (Figure 2.3).

Exposures

The interquartile range (IQR) in long-term estimates (examinations 2 and 5) was 1.9 $\mu g/m^3$ for $PM_{2.5}$ and 29.2 ppb for NO_X across all MESA study sites (Table 2.4). Estimated $PM_{2.5}$ exposure was highest in Los Angeles and New York and lowest in the Twin Cities (Figure 2.1). Estimated NO_X exposures were highest in New York and Los Angeles and lowest in Winston Salem (Figure 2.1). However, since the exposure models adjust for site, the relevant variability for these analyses is within-site.

Preadjustment of the acute exposures resulted in mean-centered estimates of detrended acute exposure (Figure 2.2). Adjustment for seasonality, temperature, and relative humidity resulted in a more symmetric distribution of exposures. The variability in acute $PM_{2.5}$ exposures was smaller at examination 5 compared to examination 2.

Interquartile ranges of the preadjusted acute exposure varied by examination and lag period (Table 2.5). Lag 0 (indicating the day of examination) and lag 1 (day prior) are more variable than the remaining lags periods which average over multiple days (lag 1-2, lag 1-3, lag 1-4, and lag 1-5).

Longitudinal Exposure Effects

Both $PM_{2.5}$ and NO_X exposures over follow-up were negatively associated with change in CRAE over time (Figure 2.4). Comparing individuals differing by 5 $\mu g/m^3$ of $PM_{2.5}$ exposure averaged over the follow-up period between their examinations 2 and 5, change in CRAE differed on average by -1.41 µm per (95% CI: -3.40, 0.58, p=0.17) (adjusting for variables from the *fully adjusted model*). Comparing individuals differing by 29.2 ppb of NO_X exposure

averaged over the follow-up period, change in CRAE differed on average by -0.69 μm per 7.8 years (95% CI: -2.04, 0.66, p=0.32) (from the *fully adjusted model*). CRAE results were similar between the adjustment approaches for both pollutants.

$PM_{2.5}$ exposure over follow-up had no association with change in CRVE, whereas NO_X exposure was negatively correlated with CRVE (Figure 2.5). Comparing individuals differing by 65 $\mu g/m^3$ of $PM_{2.5}$ exposure averaged over the follow-up period between their examinations 2 and 5, their measured change in CRVE differed on average by 0.26 μm per 7.8 years (95% CI: -2.28, 2.79, p=0.84) (from the *fully adjusted model*). Comparing differing by 29.2 ppb of NO_X exposure averaged over the follow-up period, their measured change in CRVE differed on average by -0.66 μm per 7.8 years (95% CI: -2.35, 1.03, p=0.44) (from the *fully adjusted model*).

City-Specific Longitudinal Effects

The city-specific effects of long-term $PM_{2.5}$ exposure on change in CRAE were consistent with the overall effect. Overall they were negative or null, although the magnitude varied substantially by city/study site (Figure 2.6). The city-specific effects of NO_X on CRAE varied in both magnitude and direction, but these effects were all consistent with the overall effect in terms of confidence intervals (Figure 2.7).

Interaction by Age, Gender, and Anti-hypertensives

Results for the longitudinal interaction analysis are presented in Figure 2.8 ($PM_{2.5}$, CRAE), Figure 2.9 (NO_X, CRAE), Figure 2.10 ($PM_{2.5}$, CRVE), and Figure 2.11 (NO_X, CRVE). Individuals with hypertension who were on diuretics had a stronger negative association with $PM_{2.5}$ and NO_X for both CRAE and CRVE compared to individuals not on diuretics. Otherwise,

results did not provide any compelling suggestions of effect modification given the large confidence intervals and the number of comparisons performed.

Acute Exposure Effects

Day of examination $PM_{2.5}$ exposure was positively associated with CRAE whereas day prior $PM_{2.5}$ was negatively associated with CRAE (Figure 2.12). The negative association between $PM_{2.5}$ exposure and CRAE diminished when the acute exposure estimate was defined as multiday intervals preceding the examination. Comparing individuals differing by 5 $\mu g/m^3$ of $PM_{2.5}$ exposure on the day of examination, their measured CRAE differed on average by 0.16 µm (95% CI: 0.35, -0.04, p=0.12) when controlling for seasonality, temperature, and relative humidity (via preadjustment) and adjusting for variables in the *fully adjusted model*. Comparing individuals differing by 5 $\mu g/m^3$ of $PM_{2.5}$ exposure on the day prior to examination, their measured CRAE differed on average by -0.17 µm (95% CI: 0.02, -0.36, p=0.08) when controlling for seasonality, temperature, and relative humidity via preadjustment and adjusting for variables in the *fully adjusted model*. We observed no association between acute $PM_{2.5}$ exposure and CRVE.

2.4 DISCUSSION

Very few studies exist describing longitudinal changes in retinal measures. We found suggestive evidence of negative association between long-term exposure to $PM_{2.5}$ and change over time in CRAE adjusting for cardiovascular risk factors. This effect size was relatively large compared to the average unadjusted change over time in CRAE: CRAE decreased on average by 3 µm per 7.8 years, compared to an estimated effect per 5 $\mu g/m^3$ of $PM_{2.5}$ of -1.41 µm per 7.8

years (95% CI: -3.40, 0.58, p=0.17). This confidence interval is also consistent with no effect so we cannot rule out that this result is due to random variability. NO_X was also negatively associated with both change in CRAE and change in CRVE, but these associations were approximately half as strong and, unlike for $PM_{2.5}$, the confidence intervals were not consistent with large estimates of association. On the acute time-scale, day of exam $PM_{2.5}$ exposure had a near-significant positive association with CRAE and day prior to exam $PM_{2.5}$ had a near-significant negative association with CRAE, adjusting for season and meteorology and cardiovascular risk factors. CRVE was associated with neither long-term NO_X and $PM_{2.5}$ nor acute $PM_{2.5}$ exposure.

Longitudinal Change in Retinal Measures

To our knowledge, there have been no studies of air pollution exposure and longitudinal changes in retinal vessel diameters, and very few studies on longitudinal changes in retinal vessel diameters in relation to any exposures. One longitudinal study of retinal venular diameter reported age-related changes in retinal caliber (Myers et al., 2012), and to our knowledge there are no longitudinal studies of retinal arteriolar diameter. Conventional understanding of retinal arteriolar diameters is that they tend to decrease with age. While we found this to be generally true in MESA, retinal arteriolar diameters did increase from examination 2 to examination 5 in more than a quarter of the participants over the follow-up period. Arteriolar diameter varies on a daily time-scale (Couper et al., 2002), and therefore random variation in the retinal measures could explain these increases to some extent. Alternatively, this finding could imply that at least some individuals do experience true underlying widening of basal vessel tone, although we do not know whether this is a change associated with health behaviors (for example from alterations

in medication, diet, or exercise) or an atypical form of vascular damage. Typically, narrowing of the lumen in larger vessels may reflect pathologic vascular remodeling associated with hypertension (Renna et al., 2013), and narrower arterioles are typically associated with poor health outcomes (Seidelmann et al., 2016). To our knowledge there are no studies on whether change over time in retinal vessel diameter predicts cardiovascular events, likely due to the long follow-up periods necessary to answer this question. Further epidemiologic research into the significance of retinal changes over time, for both widening and narrowing, is needed.

We observed that retinal venules diameters narrowed over time for most participants. Age-related retinal venular narrowing was previously found in a 15 year longitudinal analysis of CRVE in a Wisconsin cohort (Myers et al., 2012). While literature has found that wider retinal venules may be associated with various cardiovascular health outcomes (McGeechan et al., 2009; Seidelmann et al., 2016), these studies only show that a single baseline retinal measurement of retinal venules are related to clinical outcomes. We also observed that changes in CRAE and changes in CRVE were mildly positively correlated over time. This positive correlation is somewhat surprising given the substantial evidence showing that baseline CRAE is negatively associated with cardiovascular events and baseline CRVE is positive associated with cardiovascular events. It is apparent that the current understanding of these retinal measures is somewhat limited given that most previous analyses have not considered change over time in these measures. Further research is needed to determine the complex interrelationship of these two variables, how they change over time, and the relationship between longitudinal change in retinal measures and cardiovascular disease.

Interaction by Age, Gender, and Anti-hypertensives

Individuals with hypertension who were on diuretics had a stronger negative association between both pollutants and change in both retinal measures. A major limitation of this analysis is that it only considers use of antihypertensive medication at examination 2 and does not consider changes in antihypertensive use over follow-up. Correlation between examination 2 and examination 5 antihypertensive use was only moderately strong (Pearson's r=0.57) with a tendency for increased usage over time. Further research is needed to identify whether specific patterns of antihypertensive use over time medications may modify the observed associations, but this study may not be powered for such analyses.

Sensitivity Analyses

Estimates of change in retinal measure for differences in air pollution exposure differed only slightly between the minimally adjusted and fully adjusted models. We designed the adjustment approach in the fully-adjusted model to allow for both time-varying estimates and progression estimates (with the exception of variables introduced in the baseline model which were unlikely or impossible to be time-varying: baseline age, gender, race, education, study site, and year-prior exposure). This approach allowed complete adjustment for any possible time-related effects of these variables. We also considered simpler models that included either time-varying only adjustment or progression-only adjustment, but these did not differ appreciably (results not shown). The effect modification models for long-term exposure included only progression terms due to concerns regarding the interpretation of a model that mutually adjusts for exposures over different exposure periods that are derived from different exposure estimation approaches.

City-specific associations for the longitudinal analysis were varied but unremarkable given the uncertainty in estimation of these associations, indicating that no one city has an undue influence on the longitudinal effect estimates.

Acute Exposure and Cross-sectional Differences in Retinal Measures

Established literature on CRAE has generally held that narrower retinal arteriolar vessels are associated with poor health outcomes. We were surprised to find a relatively strong positive association between acute day of examination $PM_{2.5}$ exposure and CRAE. A controlled diesel exposure study at the University of Washington diesel chamber lab showed similar paradoxical widening of the CRAE immediately after air pollution exposure, consistent with our finding in this study (*unpublished*). However these results must be interpreted cautiously because they are not controlled for long-term exposure. Furthermore, day of exposure acute $PM_{2.5}$ corresponds to the 24 hour period (midnight to midnight) on the day of examination, and examinations were typically performed in the morning. Nevertheless, our results are not implausible if the observed associations were to be caused by exposure during the participant's morning commute.

An additional consideration for this analysis is the comparison with a previous analysis of this data that only contained MESA examination 2 measurements. This previous analysis showed a nonsignificant but slightly negative association between day prior to examination $PM_{2.5}$ and CRAE (−0.11 μm 95% CI −0.33 to 0.17 for a 5 μg/m3 increase in PM2.5 exposure) (Adar et al., 2010). This is consistent with our finding of 0.16 μm (95% CI: 0.35, -0.04, p=0.12) for the same $PM_{2.5}$ interval. However, this previous analysis did not find a positive association for day of examination. In-depth comparisons between our approach and that of Adar et al. show that the difference in estimates of association are attributable primarily to two main differences in data

and modeling decisions. The Adar et al. analysis used monitoring data from a different location for one of the cities (Winston-Salem), which contributed to some difference between estimates of association. Pre-adjustment, rather than in-model adjustment, contributed to most of the remainder of the difference. We believe the pre-adjustment is a better approach because it more efficiently estimates the association between air pollution and meteorological changes. Differences in other adjustment variables included in our respective models, by comparison, made relatively little difference in the size of the estimates of association.

Other studies on retinal vessel diameters and acute air pollution exposures have found a negative association between CRAE and PM_{10} in the 24 hours preceding retinal photography (-0.93-µm 95% CI: -1.42, -0.45 per 10-µg/m3 increase in PM_{10}) (Louwies et al., 2013). This negative relationship was attenuated, but not positive, for shorter time periods (i.e. hours) preceding retinal photographs. Since this study focused on PM_{10} the results and effect sizes are not directly comparable.

Potential Biological Mechanisms

Short-term changes in vascular diameter are regulated by contractility of vascular smooth muscle cells, present in all vessels other than capillaries. Arterioles are the primary resistance vessels; they independently control blood flow to separate organ systems, respond to systemic changes in blood pressure, and may be implicated in hypertensive pathogenesis. Typical systemic vessel diameters are regulated through the action of local or neural factors on vascular smooth muscle cell tone. However, retinal vessels lack autonomic innervation and are therefore regulated primarily through local factors (Delaey and Van De Voorde, 2000). Primary local agents which affect vasoreactivity are nitric oxide (vasodilator) and endothelin (vasoconstrictor),

and possibly factors which may be unique to the retina (Delaey and Voorde, 1998). Given the design of our study we cannot conclusively determine through which of these pathways air pollution might act.

Inflammatory cascades could inhibit local nitric oxide production, potentially causing vasoconstriction. Endothelial nitric oxide synthase (eNOS) is inhibited by oxidative stress (Montezano and Touyz, 2012). On the other hand, particulate matter may upregulate nuclear factor kappa beta (NF-kB) transcription which promotes nitric oxide production via inducible nitric oxide synthase (iNOS). Additionally, oxidative stress as measured by increased glutathione peroxidase is associated specifically with wider retinal arterioles (Daien et al., 2013). Given the complex interrelationships between reactive oxygen species, vasodilators, vasoconstrictors, and their regulatory pathways it is not possible to uniquely identify from this data a specific agent that could explain a negative association between $PM_{2.5}$ exposure and retinal arteriolar diameters. Nevertheless, it is plausible that air pollution induced changes in oxidative stress which could in turn disrupt autoregulation of vessel tone leading to changes in retinal arteriolar diameters.

The cell types through which particulate matter might alter retinal vessel tone are unknown. Previous research has identified three potential mechanisms by which air pollution might alter the vasculature: directly through particle infiltration into the blood stream, indirectly through pulmonary inflammation that releases circulating inflammatory factors, or through an autonomic response initiated in pulmonary nociceptors (Brook et al., 2002). If air pollution induces inflammation in the lungs, that could result in a systemic inflammatory cascade that alters vascular endothelial cells. This inflammatory cascade effect might not be instantaneous, so this could explain the delayed vasoconstriction seen in the possible relationship between day prior $PM_{2.5}$ exposure and CRAE. Previous studies of CRAE and acute air pollution exposure

found stronger negative effects for day prior exposure compared to exposure on the day of the retinal measurement (Adar et al., 2010; Louwies et al., 2013).

It is difficult to determine what could cause a relationship between day of examination $PM_{2.5}$ exposure and CRAE widening, but this result is consistent with our unpublished controlled exposure data. Air pollution exposure causes acute increases blood pressure likely through autonomic response via nociceptors in the lungs (Cosselman et al., 2012), so retinal vessel dilation could be the result of incomplete or delayed compensatory autoregulation in response to increased blood pressure. Alternatively, apparent retinal vasodilation could be the result of retinal vessels uniquely lacking functional sympathetic innervation (Delaey and Van De Voorde, 2000). That is, if air pollution induced blood pressure increases are mediated by sympathetic activation of systemic microvascular endothelial cells leading to systemic vascular smooth muscle cell contraction, a systemic compensatory response could release circulating vasodilators which may act on retinal vessels. In the absence of the initial sympathetic vasoconstriction, the immediate response in retinal vessels might be to respond only to the circulation compensatory vasodilating factors. Given the unique status of retinal vessels not receiving sympathetic innervation, this proposed mechanism merits further research.

Relatively little is known about the pathobiologic significance of changes in venular tone compared to arteriolar tone. Whereas arterial vascular tone is primarily involved in regulation of blood flow to local tissue and mediating arterial pressure, changes in venular tone determine central venous pressure which influences venous volume and cardiac filling. Changes in retinal venular diameters and arteriolar diameters may represent different biologic processes: narrower arteriolar diameter tends to be strongly related to hypertension (Leung et al., 2004; Wong et al., 2004), whereas larger venular diameter may represent inflammatory processes (Klein et al.,

2006). Given that venous pressure is a less common clinical measure (compared to arterial pressure), risk factors for changes in venular tone and associated diseases are less well understood than for the arterioles. Retinal venular diameters have been previously associated with inflammatory markers (Sabanayagam et al., 2015), but relatively little is known about their pathobiology in relation to clinical cardiovascular disease despite well-replicated epidemiologic associations with cerebrovascular disease and cardiovascular events (Doubal et al., 2009; Guo et al., 2016; Ikram et al., 2006; Wong et al., 2006b). Venous vessels have relatively little basal tone compared to arteriolar vessels and therefore are normally in a more dilated state, so accumulation of vasodilators has minimal effect on venules. Given that previous epidemiologic studies only relate venule diameter at a single time point to cardiovascular disease, it is impossible to determine whether the association between wider venules and clinical events is the result of actual widening over time of venules or person-to-person variability in venular diameter. However, given the relatively low basal tone of venules, if air pollution affects microvascular diameter through alteration of vessel tone then it is unsurprising that we observed less strong associations between acute exposures and CRVE.

Possible Distinct Long-term effects

Changes in retinal vessel diameters may reflect a combination of acute, reversible processes and long-term processes which are more permanent. Relatively little research has addressed the question of whether long-term changes in vessel tone reflect a different mechanism from alteration of vessel tone through typical acute pathways. Some authors have argued that retinal vessel diameter reflects vascular remodeling in the microvasculature (Cheung et al., 2007; Ding et al., 2014). The process of vascular remodeling is understood to be related to the chronic

condition of hypertension, although it is still unknown whether vascular changes cause or are a side effect of hypertension. Vessel wall thickening and semi-permanent alterations in vessel tone (e.g. through vascular smooth muscle cell hypertrophy) in response to extraneous increases in blood pressure could result in a feedback loop which amplifies or solidifies increases in blood pressure.

Alteration of vessel diameter is one of several mechanisms that theoretically could explain the relationship between air pollution exposure and cardiovascular disease events. Statistically, CRAE and other measures of vessel health are associated with risk of future clinical cardiovascular events. In a cohort of patients followed for an average of 28 months, baseline endothelial dysfunction was found to predict risk of cardiac events in a cohort of patients with mild coronary artery disease (Suwaidi et al., 2000). Mechanistically, narrower vessels with thicker walls can exacerbate angina, reduced vessel reactivity can increase the likelihood of a clot causing occlusions, and altered regulation of vessel tone could lead to permanent increases in blood pressure. If air pollution affects microvasculature of the heart, it may therefore exacerbate coronary artery disease. Further research is needed to determine whether alteration in vessel tone may mediate the relationship between air pollution exposure and cardiovascular events.

Strengths and Limitations

Various sources of bias could explain the apparent associations in this study. We cannot rule out associations due to random chance, although to some extent our results replicate previous findings which makes this less likely. As always, we cannot rule out bias due to unmeasured confounding or loss-to-follow-up. Additionally, acute and long-term effects may

not be separately identifiable. Due to differences in how the acute and long-term estimates were estimated, we did not fit a model containing both acute and long-term exposures due to concerns that it would not correctly estimate separate acute and long-term effects. Therefore, we used separate models for acute and long-term exposure rather than mutual adjustment. In the presence of a positive correlation between these exposures and if there is a true effect of air pollution on only one time-scale, we would expect to estimate statistical associations in both time scales using this approach. That is to say, the effect of acute exposure could potentially be confounded by long-term exposure or vice versa. However, it may be unlikely that there is a relationship between the sources of exposure contrasts because acute exposure contrast derives from temporal variation due to differences in examination dates, whereas long-term exposure contrast derives from spatial variation from residence location. The date of participant examination is unlikely to be related to place of residence for later examinations where spatial recruitment patterns are more diluted by random variability in scheduling. In the absence of a relationship between these sources of exposure contrast, acute and long-term effects would be separately identifiable.

A major limitation of this study is the fact that retinal vessels may not necessarily represent systemic microcirculation. Unlike typical microvessel, retinal vessels do not receive sympathetic innervation (Delaey and Van De Voorde, 2000). It has also been hypothesized that there may be a local vasodilating factor unique to the retinal vasculatures (Delaey and Voorde, 1998; Maenhaut et al., 2007). While our study has shown a possible association between air pollution and retinal arteriolar tone, the results cannot be easily generalized to other vessels.

Strengths of this study include its large sample size, prospective data collection, and well-validated exposure measures. Additionally, the large number of participant characteristics collected allowed us to control for a number of confounding variables, reducing the likelihood of

unmeasured confounding. We adjusted for study site in each of our models, eliminating the possibility of confounding by recruitment center or region.

We found strong negative associations between both acute day prior and long-term $PM_{2.5}$ associations with CRAE which replicated previous cross-sectional acute and long-term results. The association for 5 $\mu g/m^3$ long-term $PM_{2.5}$ exposure was equivalent to about half the average change in CRAE over the 7.8 years of follow-up in MESA participants. We additionally found a paradoxical associations between acute day of examination $PM_{2.5}$ and CRAE that may replicate unpublished diesel chamber results. For both acute and long-term exposures, the uncertainty in the estimates of association indicates that these results are consistent with either strong associations or no relationship, so further research in larger studies is necessary. Overall, the results suggest a there may be relationship between exposure to particulate matter and vascular changes. While the unique physiology of retinal vessels limits the precise interpretation of these results with respect to systemic and coronary circulation is unknown, these results could indicate that air pollution could cause vascular changes that are not directly mediated by sympathetic activation. Further research is needed to investigate biologic pathways that might mediate a relationship between air pollution exposure and vessel diameters, and more broadly to determine whether vascular changes mediate the relationship between air pollution exposure and cardiovascular disease.

2.5 FIGURES

Figure 2.1. Estimated Long-term Exposure Distributions by Study Site

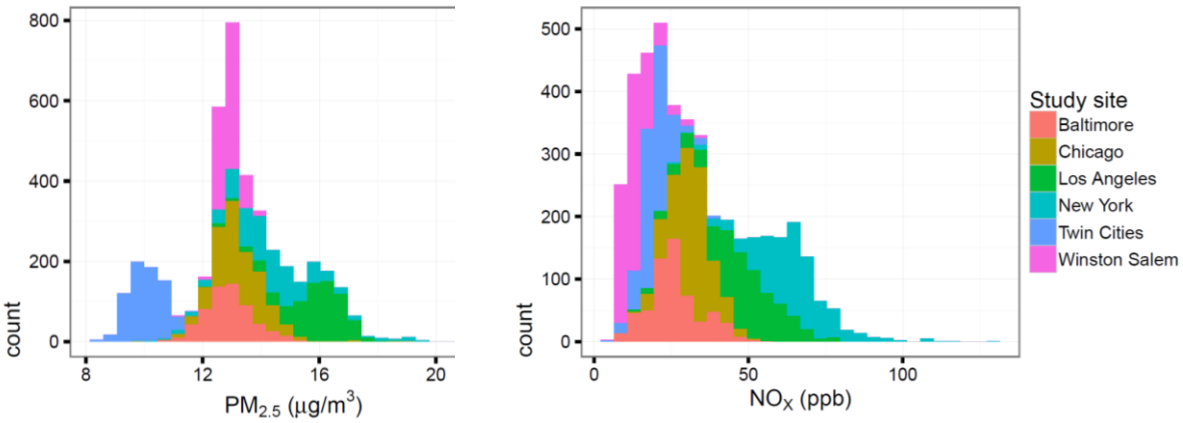
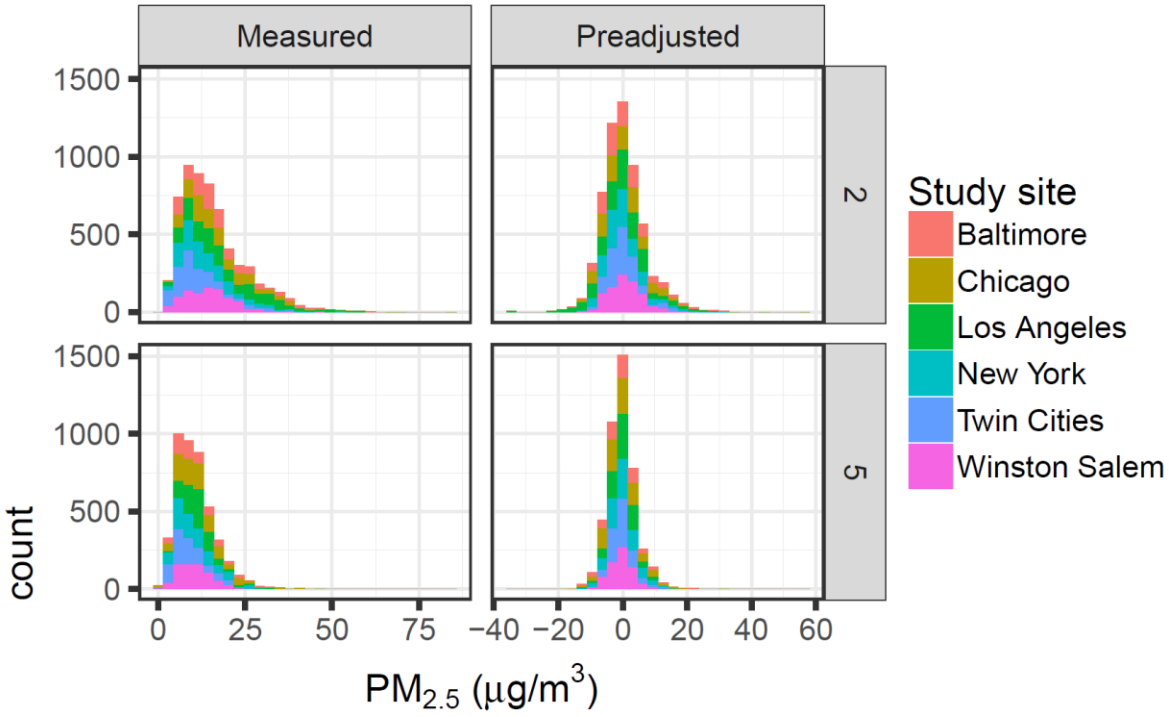


Figure 2.2. Distribution of Acute Preadjusted $PM_{2.5}$ Exposures by Study Site and Examination.



Acute exposure from central monitors on day of examination. Rows correspond to examination period (examination 2 and 5) and columns indicate raw, measured measurements versus the preadjusted measurements. Values were preadjusted with separate models by site, resulting in mean centered values.

Figure 2.3. Relationship between Longitudinal Change in CRAE and Change in CRVE in MESA

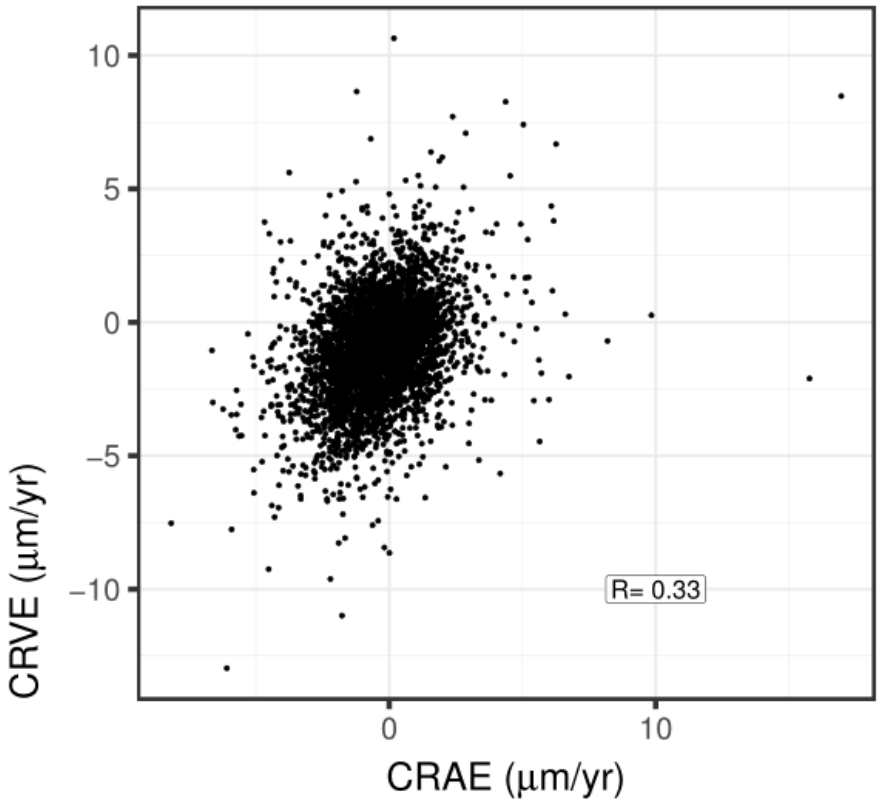
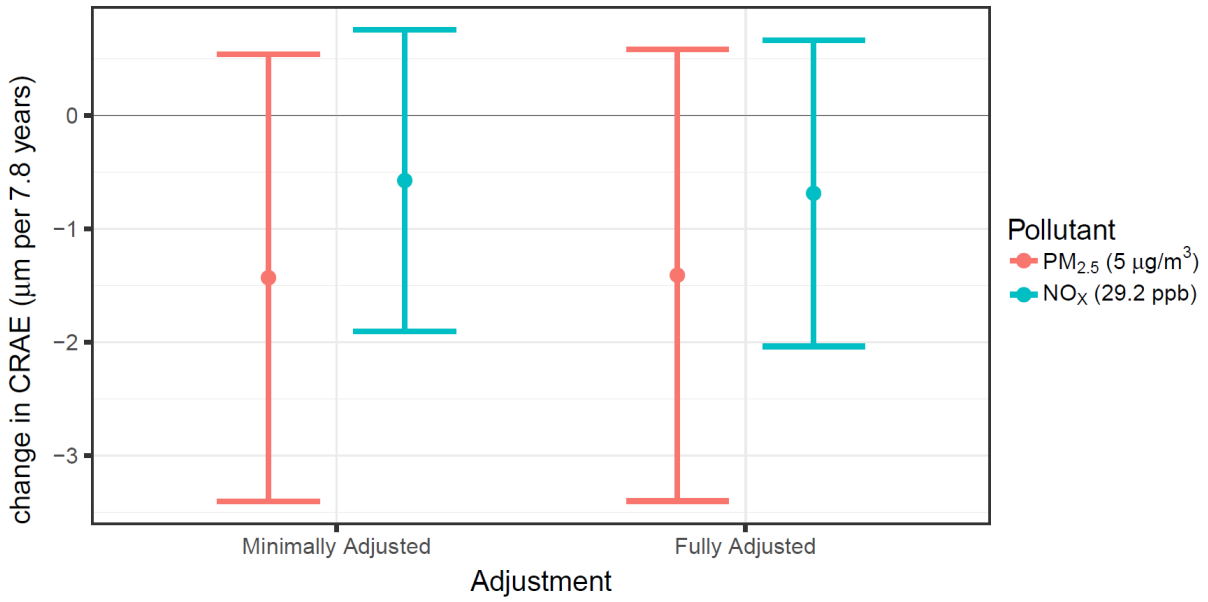
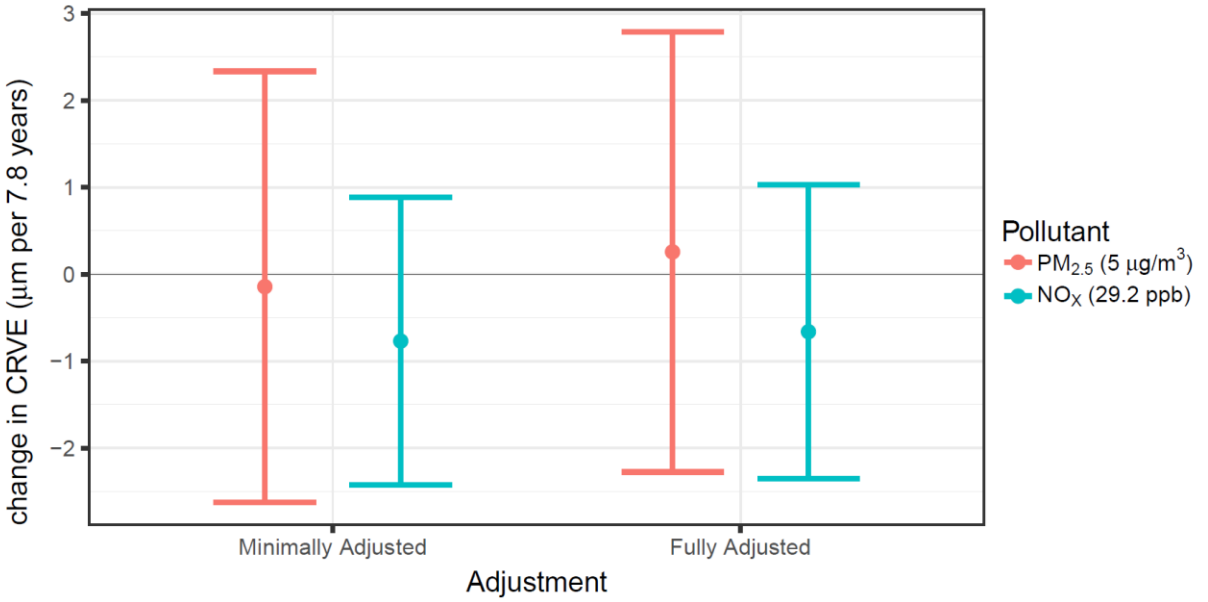


Figure 2.4. Long-term Air pollution Exposure and Change in CRAE



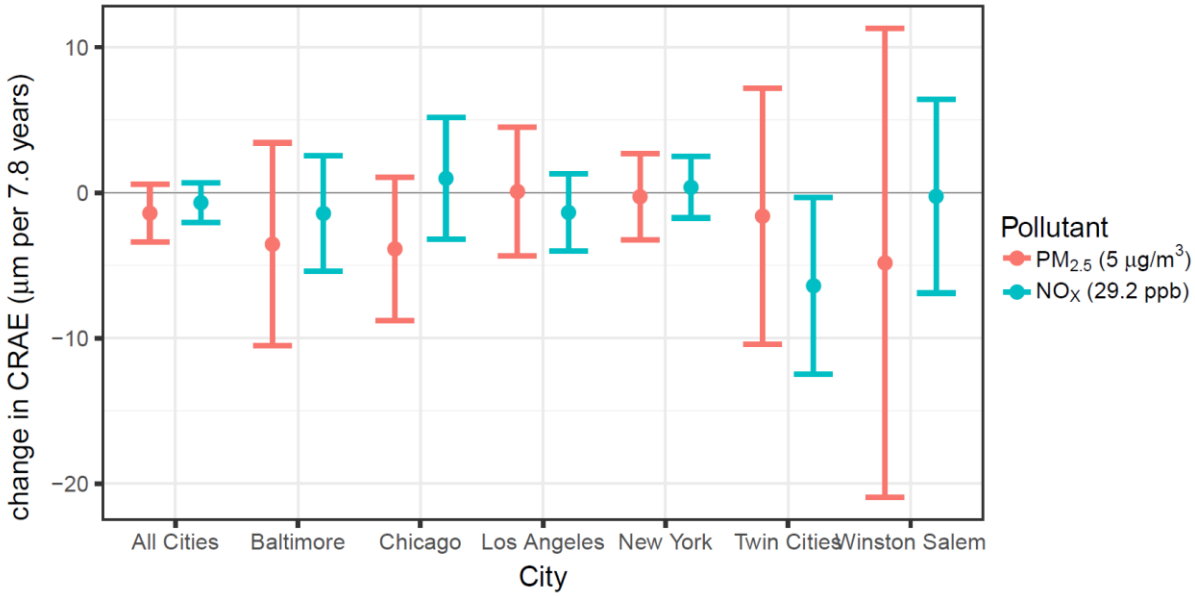
The "Minimally adjusted" model includes adjustment for progression due to baseline age, gender, race, education, site, and year-prior exposure. The "Fully Adjusted" model includes "Minimally Adjusted" terms plus progression adjustment for baseline (examination 2) and time-varying adjustment (examinations 2 and 5) for the following variables: smoking status, BMI, diastolic blood pressure, systolic blood pressure, diabetes status, weekly intentional exercise, anti-hypertensive medications, lipid-lowering medications.

Figure 2.5. Long-term Air pollution Exposure and Change in CRVE



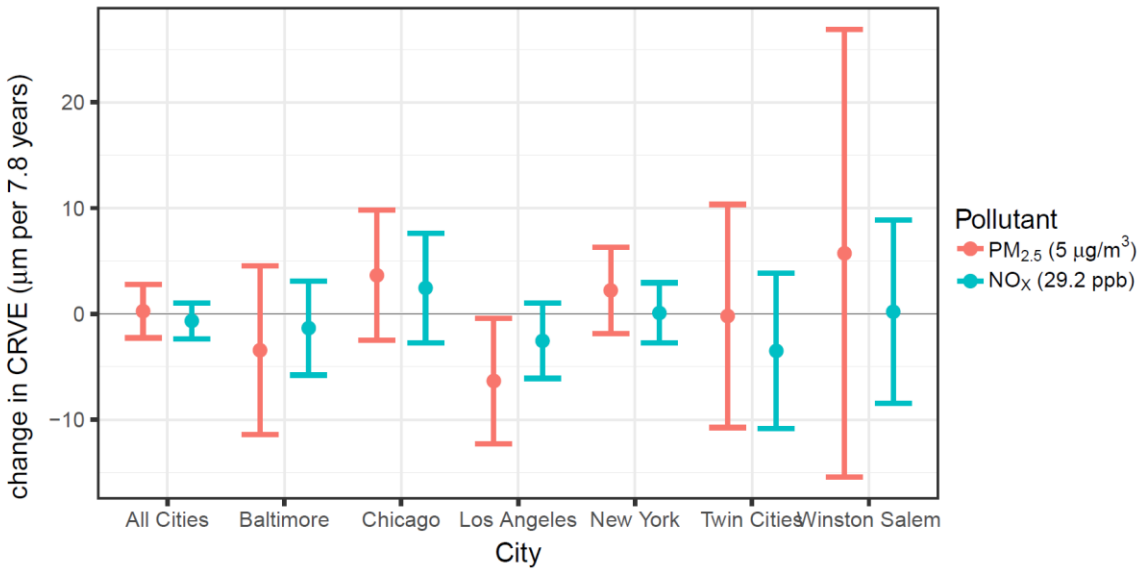
The "Minimally adjusted" model includes adjustment for progression due to baseline age, gender, race, education, site, and year-prior exposure. The "Fully Adjusted" model includes "Minimally Adjusted" terms plus progression adjustment for baseline (examination 2) and time-varying adjustment (examinations 2 and 5) for the following variables: smoking status, BMI, diastolic blood pressure, systolic blood pressure, diabetes status, weekly intentional exercise, anti-hypertensive medications, lipid-lowering medications.

Figure 2.6. City-specific Associations of Long-term Air Pollution with Changes in CRAE



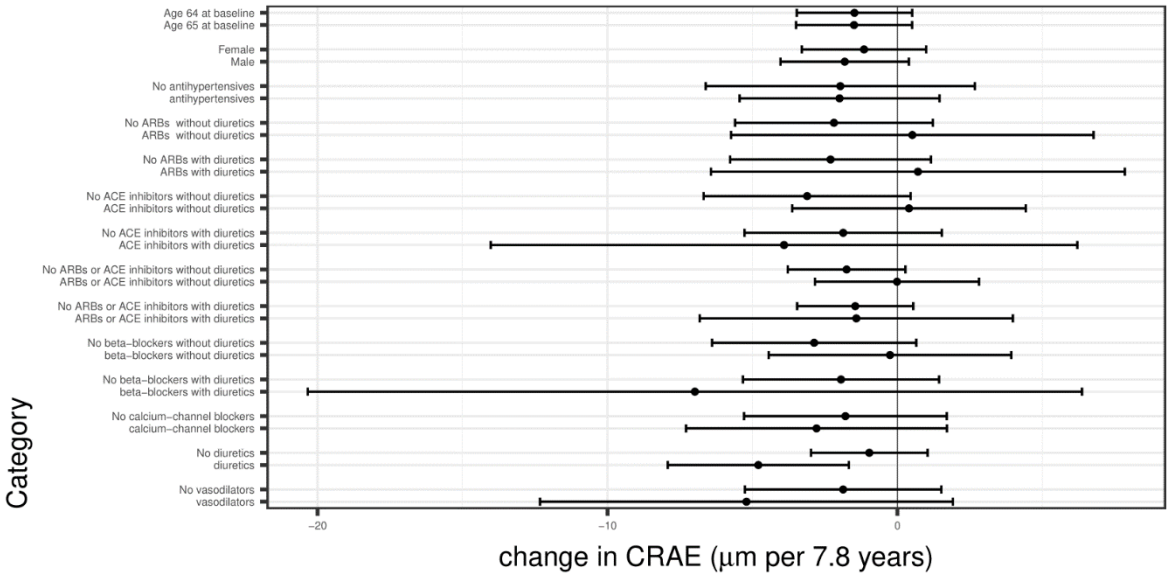
City-specific results are from separate (stratified) models. These are "fully adjusted" models which include adjustment for progression due to baseline age, gender, race, education, site, and year-prior exposure. These models additionally include both progression adjustment for baseline (examination 2) and time-varying adjustment (examinations 2 and 5) for the following variables: smoking status, BMI, diastolic blood pressure, systolic blood pressure, diabetes status, weekly intentional exercise, anti-hypertensive medications, lipid-lowering medications. The "All Cities" model additionally includes progression adjustment for site.

Figure 2.7. City-specific Associations of Long-term Air Pollution with Changes in CRVE



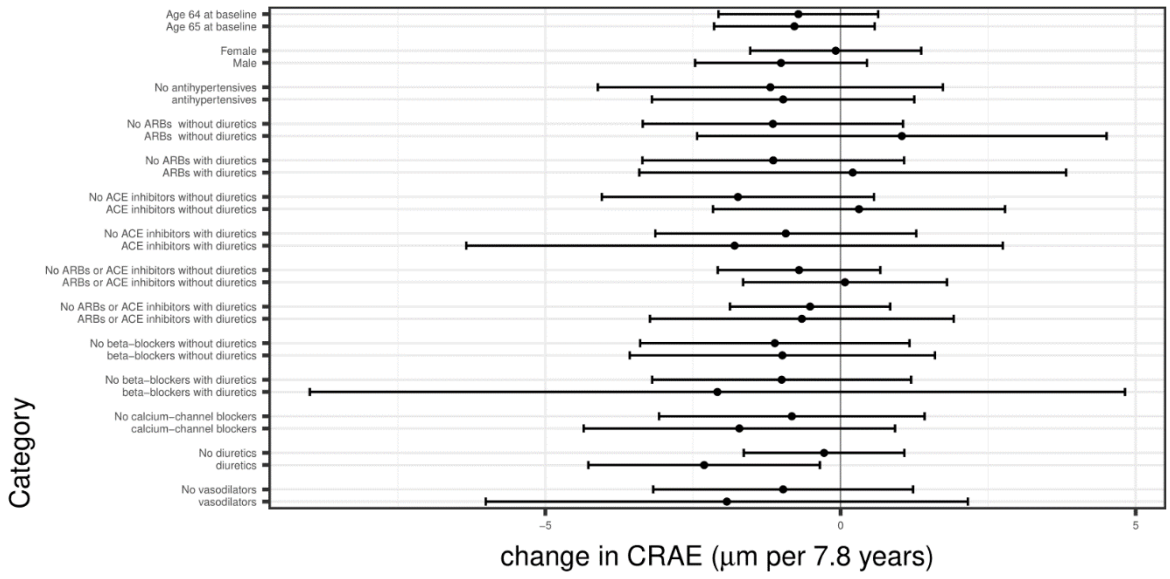
City-specific results are from separate (stratified) models. These are "fully adjusted" models which include adjustment for progression due to baseline age, gender, race, education, site, and year-prior exposure. These models additionally include both progression adjustment for baseline (examination 2) and time-varying adjustment (examinations 2 and 5) for the following variables: smoking status, BMI, diastolic blood pressure, systolic blood pressure, diabetes status, weekly intentional exercise, anti-hypertensive medications, lipid-lowering medications. The "All Cities" model additionally includes progression adjustment for site.

Figure 2.8. Long-term $PM_{2.5}$ Exposure and Change in CRAE: Age, Gender, and Antihypertensive Specific Results



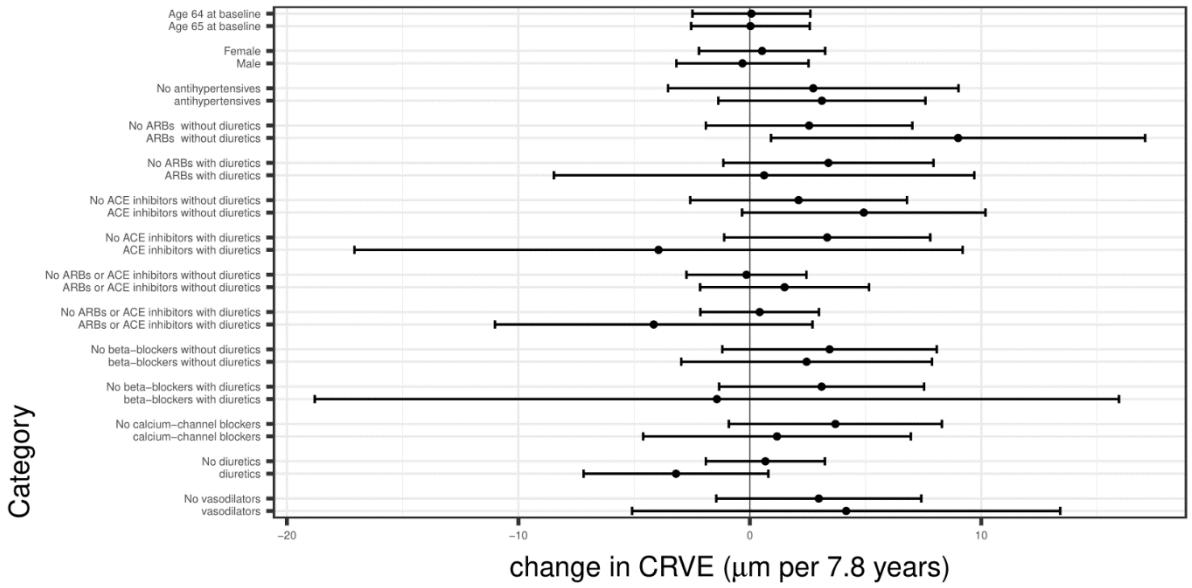
For individuals differing by 5 $\mu g/m^3$ of $PM_{2.5}$ exposure.

Figure 2.9. Long-term NO_X Exposure and Change in CRAE: Age, Gender, and Antihypertensive Specific Results



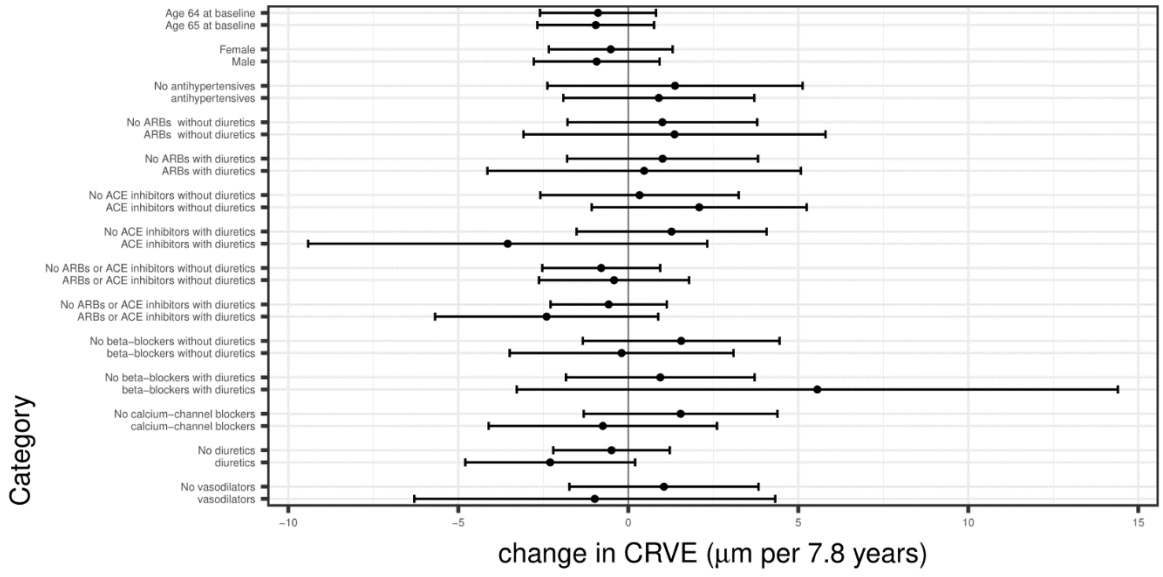
For individuals differing by 29.2 ppb of NO_2 exposure.

Figure 2.10. Long-term $PM_{2.5}$ Exposure and Change in CRVE: Age, Gender, and Antihypertensive Specific Results



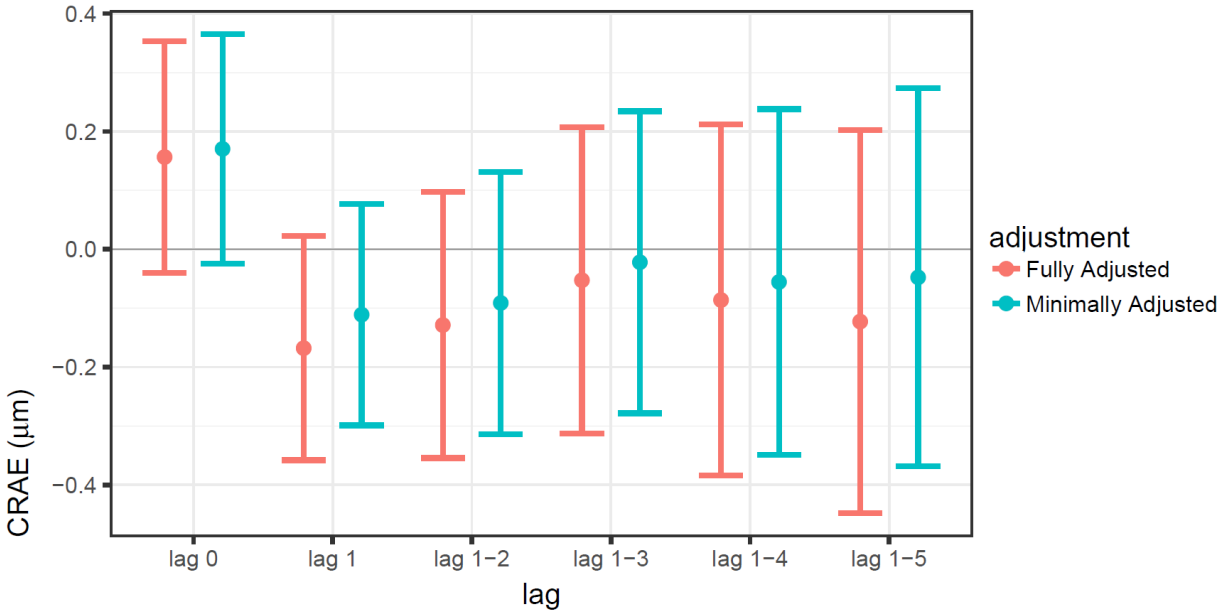
For individuals differing by 5 $\mu g/m^3$ of $PM_{2.5}$ exposure.

Figure 2.11. Long-term NO_X Exposure and Change in CRVE: Age, Gender, and Antihypertensive Specific Results



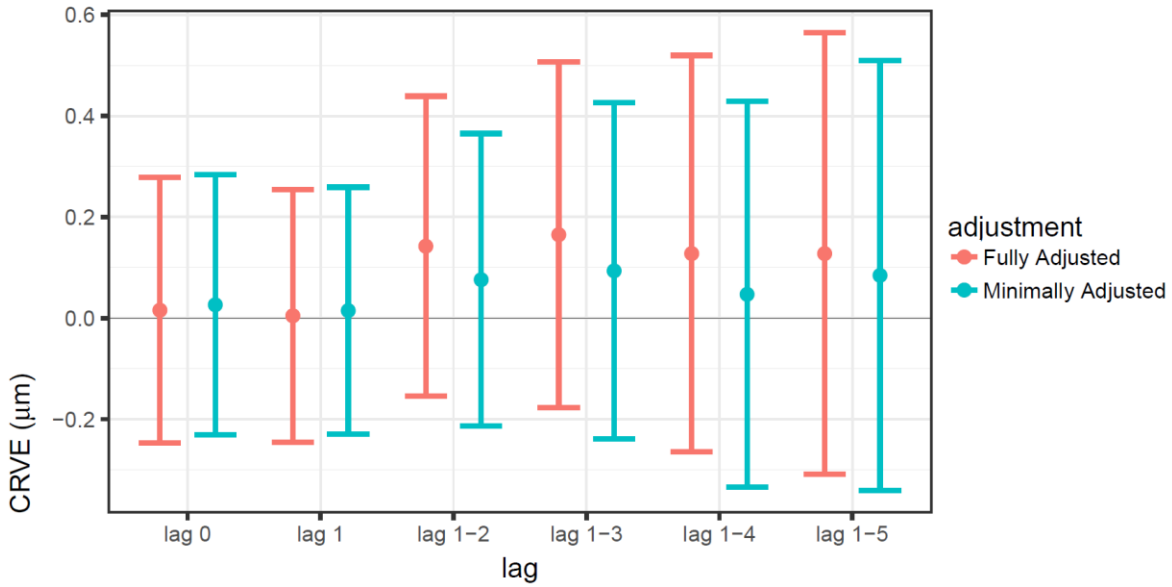
For individuals differing by 29.2 ppb of NO_2 exposure.

Figure 2.12. Association between Acute $PM_{2.5}$ Exposure and CRAE



For a 5 $\mu g/m^3$ difference in acute $PM_{2.5}$ exposure. Minimally adjusted models include adjustment for progression due to baseline age, gender, race, education, and site. Fully adjusted models include minimal adjustment terms and adjustment for both progression adjustment for baseline (examination 2) and time-varying adjustment (examinations 2 and 5) for the following variables: smoking status, BMI, diastolic blood pressure, systolic blood pressure, diabetes status, weekly intentional exercise, anti-hypertensive medications, lipid-lowering medications.

Figure 2.13. Association between Acute $PM_{2.5}$ Exposure and CRVE



For a 5 µg/m^3 difference in acute $PM_{2.5}$ exposure. Minimally adjusted models include adjustment for progression due to baseline age, gender, race, education, and site. Fully adjusted models include minimal adjustment terms and adjustment for both progression adjustment for baseline (examination 2) and time-varying adjustment (examinations 2 and 5) for the following variables: smoking status, BMI, diastolic blood pressure, systolic blood pressure, diabetes status, weekly intentional exercise, anti-hypertensive medications, lipid-lowering medications.

2.6 TABLES

Table 2.1. MESA Participant Characteristics by Follow-up Status

	Exam 1	Exam 2 Follow-up	Exam 2 CRAE*	Exam 5 CRAE**
N	6814	6233	5921	3988
CRAE at Exam 2, μm			144 (14)	145 (14)
CRAE at time of Exam, μm			144 (14)	141 (15)
CRVE at Exam 2, μm			214 (22)	214 (21)
CRVE at time of Exam, μm			214 (22)	206 (22)
Age at baseline, y	62.2 (10.2)	62 (10.2)	61.5 (10)	59.4 (9.2)
Age at time of exam, y	62.2 (10.2)	63.6 (10.1)	63.1 (9.9)	68.8 (9.1)
Gender				
Male	3213 (47%)	2969 (48%)	2834 (48%)	1888 (47%)
Female	3601 (53%)	3264 (52%)	3087 (52%)	2100 (53%)
Race/ethnicity				
White	2622 (38%)	2464 (40%)	2352 (40%)	1645 (41%)
Chinese	803 (12%)	728 (12%)	694 (12%)	477 (12%)
Black	1893 (28%)	1691 (27%)	1593 (27%)	998 (25%)
Hispanic	1496 (22%)	1350 (22%)	1282 (22%)	868 (22%)
Education				
Less than high school	1225 (18%)	1044 (17%)	974 (16%)	524 (13%)
High school	1236 (18%)	1124 (18%)	1054 (18%)	676 (17%)
Some college/technical	1937 (29%)	1773 (29%)	1689 (29%)	1182 (30%)
College or graduate	2393 (35%)	2274 (37%)	2187 (37%)	1601 (40%)
BMI at baseline, kg/m^2	28.3 (5.5)	28.3 (5.4)	28.3 (5.4)	28.3 (5.4)
BMI at time of exam, kg/m^2	28.3 (5.5)	28.4 (5.5)	28.4 (5.5)	28.5 (5.6)
Smoking at baseline				
Never	3418 (50%)	3142 (51%)	2991 (51%)	2046 (51%)
Former	2487 (37%)	2293 (37%)	2161 (37%)	1446 (36%)
Current	887 (13%)	781 (13%)	753 (13%)	491 (12%)
Smoking at time of exam				
Never	3418 (50%)	2857 (46%)	2721 (46%)	1786 (45%)
Former	2487 (37%)	2637 (43%)	2490 (42%)	1861 (47%)
Current	887 (13%)	695 (11%)	671 (11%)	311 (08%)

Summary values are mean (SD) or count (percentage). "At time of exam" corresponds to the examination indicated in the column title. *All participants for whom CRAE was nonmissing at exam 2 **Participants for whom CRAE measures was nonmissing at both exams

Table 2.2. Sample Sizes and Sources of Missingness

	n (CRAE)	n (CRVE)
Baseline	6814	6814
Any follow-up at exam 2	6233	6233
Any follow-up at exams 2 & 5	4615	4615
Retinal measure at 2 & 5	3988	4031
Retinal measure and long-term $PM_{2.5}$ at exams 2 & 5	3700	3744
Retinal measure and long-term NO_X at exams 2 & 5	3692	3736
Retinal measure and acute $PM_{2.5}$ at exams 2 & 5	3596	3633

"Any follow-up" indicates the individual participated in a clinic visit during that examination period.

Table 2.3. Distribution of Individual Rates of Changes in Retinal Measures

Variable	Mean	Median	25th %ile	75th %ile
CRAE ($\mu m/7.8yr$)	-3.0	-3.2	-10.3	3.8
CRVE ($\mu m/7.8yr$)	-7.6	-7.2	-16.4	1.3

Individual difference between the follow-up retinal measure and the baseline (examination 2) retinal measure divided by individual follow-up time multiplied by the average follow-up time of 7.8 years.

Table 2.4. Distribution of Long-Term Exposure Estimates between Examinations 2 and 5

Pollutant	units	Mean	25th %ile	75th %ile	IQR
$PM_{2.5}$	$\mu g/m^3$	13.2	12.5	14.4	1.9
NO_X	ppb	34.2	18.8	48.0	29.2

For exposure estimated over the period between exams 2 and 5.

Table 2.5. Interquartile Ranges of Preadjusted Acute $PM_{2.5}$ Exposures by Examination and Lag Period ($\mu g/m^3$)

Examination	lag 0	lag 1	lag 1-2	lag 1-3	lag 1-4	lag 1-5
2	7.5	8.2	7.1	6.2	5.5	5.4
5	4.7	5.0	4.7	4.3	3.8	3.4

Chapter 3. AIR POLLUTION EXPOSURE AND ADHESION PROTEINS

3.1 INTRODUCTION

Acute exposure to air pollution may increase risk of cardiovascular events, and growing evidence suggests that chronic air pollution exposure is related to subclinical measures of atherosclerosis in otherwise healthy individuals. Additional research is needed to determine biologic mechanisms underlying these associations and to identify biomarkers signifying the effect of air pollution on the cardiovascular disease process.

Cellular adhesion describes the interaction or attachment between cellular surfaces and other cells or substrates. Alteration of adhesion processes may play a role in cardiovascular disease development (Blankenberg et al., 2003). The underlying mechanisms that regulate adhesion are multifaceted, involving multiple steps and different classes of proteins, so isolating atherogenic effects of any individual molecule is difficult. Nevertheless, studying the associations between air pollution exposure, cardiovascular disease, and markers of cellular adhesion is a potentially rewarding research area that could identify early biomarkers for the pathogenic effects of air pollution exposure.

Cellular adhesion occurs through direct cell-cell rolling and adhesion, extracellular signaling, alteration of the extracellular matrix, and regulation of cellular proliferation. The primary molecules involved in leukocyte-endothelium interactions are selectins, which promote leukocyte rolling along vessel walls, and adhesion molecules including ICAM-1, which bind the leukocytes to the vascular surface (Blankenberg et al., 2003). ICAM-1 is highly expressed in atherosclerotic lesions, and knockout evidence in mice shows that ICAM-1 may be implicated in

spontaneous lesion formation (Bourdillon et al., 2000). These adhesion molecules are expressed on the surface of endothelial cells, but their expression is regulated by a complex set of the extracellular signaling molecules including cytokines (Turner et al., 2014). Cytokines and chemokines are involved in directing leukocytes to inflammatory sites and plaque formation and development (Barlic and Murphy, 2007). Expression of cellular adhesion molecules may be related to various growth factors such as hepatocyte growth factor, which has been implicated in lesion destabilizing angiogenesis via endothelial migration and proliferation (Ma et al., 2002). Other factors involved in lesion formation, vessel wall degradation, and small vessel angiogenesis are the metalloproteinases which promote remodeling of lesions via alteration of the extracellular matrix. (Henriksen and Sallenave, 2008; Jackson and Nguyen, 1997). Although lesion formation and destabilization involve a complex set of processes and molecules, evidence suggests that adhesion-related proteins and growth factors are involved in atherosclerotic disease development.

Epidemiologic evidence implicates a number of subclinical measures including inflammation and blood pressure as mechanisms linking air pollution and cardiovascular disease. Numerous studies have demonstrated associations between air pollution and clinical cardiovascular events both on the scale of acute (Samet et al., 2000) and chronic exposures (Miller et al., 2007). More recent studies show that chronic exposure to air pollution may be associated with measures of subclinical atherosclerosis and cardiovascular disease such as coronary artery calcium (Kaufman et al., 2016), blood pressure (Chan et al., 2015), and inflammatory markers (Hajat et al., 2015). Controlled exposure studies demonstrate that diesel exhaust causes acute endothelial changes (Peretz et al., 2008) and acute blood pressure changes (Cosselman et al., 2012) in an experimental setting.

Although there is a large amount of epidemiological evidence linking air pollution exposure and cardiovascular disease, the extent to which air pollution affects adhesion processes is largely unknown. Several biological mechanisms have been proposed to explain the apparent relationship between air pollution exposure and development of cardiovascular disease (Brook et al., 2010), and these mechanisms are theoretically consistent with air pollution induced alteration of cellular adhesion. Acute particulate matter exposure causes autonomic changes. Diesel exposure induced blood pressure changes are mediated by α1-adrenoceptors (Cosselman et al., 2016). Autonomic changes may be initiated when particulate matter activates pulmonary nociceptors such as TRPV1 (Dawson et al., 2011; Sack et al., 2015). Autonomic imbalance may directly alter endothelial expression through neuroreceptors or indirectly through an inflammatory cascade of cytokines (Amiya et al., 2014; Karakas et al., 2018). Air pollution may induce reactive oxygen species (ROS) which increase expression of adhesion molecules (Rui et al., 2016). We aim to estimate the associations of chronic and acute air pollution concentration with soluble protein concentrations of a suite of adhesion-associated proteins involved in atherosclerotic vascular damage including various adhesion proteins, selectins, cytokines, growth factors, and metalloproteinases measured in serum and plasma.

3.2 METHODS

The MESA Cohort

The Multi-Ethnic Study of Atherosclerosis is a multi-center prospective cohort study designed to capture clinical and subclinical measures of cardiovascular disease from a diverse population. Participants (n= 6,814) were recruited from six different study sites (Los Angeles, California; Minneapolis-St. Paul ("Twin Cities"), Minnesota; Chicago, Illinois; Baltimore, Maryland; Winston-Salem, North Carolina; and New York, New York) over the period from

July 2000 to August 2002. Participants were white, black, Hispanic, and Chinese. Participants underwent 5 medical examinations over the course of approximately 10 years. Examinations occurred between 2000 and 2002 (examination 1), between 2002 and 2004 (examination 2), between 2003 and 2005 (examination 3), between 2005 and 2007 (examination 4), and finally between 2010 and 2012 (examination 5). An additional follow-up examination (examination 6) occurred later, from 2016-2018. At these clinic visits, subjects received diagnostic tests and completed questionnaires about health and risk factors for cardiovascular disease.

Adhesion Proteins

A set of cellular adhesion markers were measured in serum or plasma drawn at the second examination ("examination 2") in a random subset of participants (plasma n=2,536; serum n=2,403. These markers were chosen to represent a wide range of adhesion-related vascular processes. Adhesion-related proteins were quantified through enzyme-linked immunosorbent assay (ELISA). Four proteins were measured in plasma including TGFb1 (transforming growth factor beta-1), SDF1a (stromal cell-derived factor 1), CCL5 (RANTES), and P-selectin. Proteins measured in serum included HGF (Hepatic Growth Factor), VCAM-1 (vascular cell adhesion protein 1), ICAM-1 (intercellular adhesion molecule), L-selectin, E-Cadherin, MMP-1 (matrix metalloproteinase-1), MMP-2 (matrix metalloproteinase-1), TIMP-2 (tissue inhibitor of metalloproteinase), CCL21 (6Ckine), SLPI (secretory leukocyte protease inhibitor), and IL2sr (soluble interleukin 2 receptor). Collection and storage of biological samples was standardized between study centers (Bild et al., 2002) and samples were analyzed at a centralized laboratory. Analytical methods with corresponding validity statistics and minimum detectible levels are described in Table 3.1.

Measured proteins can be divided into five groups representing their known mechanisms in atherosclerotic disease development. *Leukocyte rolling* molecules include L-selectin and P-selectin. *Adhesion proteins* include E-cadherin, ICAM-1, and VCAM-1. *Metalloproteinases and protease inhibitors* are involved in building and destruction of the extracellular matrix via degradation of collagen and elastin; alteration of this matrix may promote migration and alteration of endothelial and smooth muscle cells. *Cytokines and chemokines* include CCL21, IL-2sr, CCL5, and SDF-1α. These are inflammatory signaling molecules that may promote attraction and arrest of leukocytes to endothelial lesions. *Growth factors* include HGF and TGFb-1; these may regulate regeneration, proliferation, and lipid deposition.

Exposure Estimates

To estimate the acute and chronic associations between air pollution and adhesion proteins, we first needed to estimate participant exposures from participant home addresses. We calculated $PM_{2.5}$ exposure on two separate time-scales: daily and multi-year periods. NO_X exposure was only estimated on the multi-year period and not on the daily period due to the highly variable spatial characteristics of NO_X and lack of sufficient spatial NO_X monitoring coverage on the acute time-scale.

Long-term estimates of $PM_{2.5}$ and NO_X exposure, representing multi-year exposures, were calculated using validated MESA-specific statistical models for predicting participant air pollution concentrations at their home address during the study period (Keller et al., 2015). Briefly, these exposure models utilize smooth spatial and temporal trends and incorporate cohort-specific monitoring, meteorological information, roadway dispersion modeling, and land-use characteristics such as distance from roadway to characterize fine-scale traffic-related air

pollution. We averaged participant-specific estimates over the year prior to examination 2. These long-term estimates of $PM_{2.5}$ and NO_X exposure were produced using separate statistical models.

In order to obtain daily estimates of participant exposure to $PM_{2.5}$, we used a single centrally-located EPA monitoring station for each of the six study sites as an estimate for city-wide average $PM_{2.5}$ concentrations. Using a single monitor for a city is an established approach to acute exposure assessment within the MESA study, as daily variations in $PM_{2.5}$ tend to be spatially homogenous (Adar et al., 2010). Since spatial trends in $PM_{2.5}$ are well-characterized by our long-term exposure model, these short-term city-wide pollution levels provide complementary temporal variation in order to estimate associations between acute air pollution exposure and the health outcomes. Exposure contrast for the acute analysis is temporal (rather than spatial), leveraging differences in the days that participant examinations occurred.

To eliminate the possibility of confounding by temporal factors, the complete daily time-series of monitoring data was preadjusted for meteorologic factors and time using pre-adjustment (Szpiro et al., 2014). We used polynomial splines to model the time-series of $PM_{2.5}$ monitoring data from a single Environmental Protection Agency Air Quality System monitor in each city. Separate models were fit for each monitor. Variables included were temperature (6 degrees of freedom per year), relative humidity (6 degrees of freedom per year), and seasonality (using a continuous adjustment for time with 12 degrees of freedom per year).

We used preadjusted estimates on days relative to each participant's examination dates as the exposure in the acute exposure analysis. To limit the number of comparisons given the large number of outcome variables, we selected the day prior to examination as the exposure period of interest. Estimation of daily exposure estimates of $PM_{2.5}$ did not incorporate the MESA spatiotemporal model or MESA monitoring data due to limitations of the MESA monitoring data

(fixed two week intervals are the shortest resolvable averaging period for estimates produced by the MESA-specific models).

The acute $PM_{2.5}$ exposures we employed are mean-centered for each study site as a result of pre-adjustment. Mean-centering by study site implies that the preadjusted acute $PM_{2.5}$ estimates have study site-specific averages of zero which effectively erases between city differences in $PM_{2.5}$ exposure, but all of our health effect models adjust for study site so the mean-centering does not affect estimation of acute $PM_{2.5}$ exposure on adhesion proteins.

Statistical Models

Using linear models we estimated the associations between each air pollutant (long-term NO_X and $PM_{2.5}$ and day prior $PM_{2.5}$) and each adhesion-related protein separately. We scaled the estimates of association to represent a change in the adhesion marker for a 5 $\mu g/m^3$ interval in year prior $PM_{2.5}$ exposure and for a 42.1 ppb interval in year prior NO_X exposure. The results for the acute analysis are also scaled to represent an interval of 5 $\mu g/m^3$. The interval of 5 $\mu g/m^3$ approximates the interquartile range for year-prior $PM_{2.5}$ exposure in the full MESA cohort at examination 2 (4.9 $\mu g/m^3$) and day prior exposure (8.2 $\mu g/m^3$), and the interval of 42.1 ppb is the interquartile range for year-prior NO_X exposure at MESA examination 2. Due to the number of comparisons, we used a Bonferroni adjustment for multiple comparison (α=0.05/30 or 0.0017).

Confounder Adjustment

We used a staged approach for confounding adjustment. Potential confounders incorporated into successive models based on *a priori* knowledge and epidemiologic research. Model 1 represents likely confounders and includes age, gender, race, site, smoking status

(current, former, never), education (no school, grades 1-8, grades 9-11, high school/GED, some college, technical school, associate degree, bachelor's degree, graduate or professional school), and BMI (continuous). Model 2 included the variables from model 1 as well as diastolic blood pressure (continuous), systolic blood pressure (continuous), diabetes (normal, impaired, untreated diabetes, treated diabetes), weekly intentional exercise (continuous), lipid lowering medication (yes/no), and alcohol use (yes/no). Because models were adjusted for study site, exposure contrast for long-term estimates of air pollution came from within city variation.

We performed an analysis for effect modification. We estimated interaction effects by smoking status (current, former, never) and BMI categories (BMI <25, Normal weight; BMI >25-30, Grade 1 Overweight; BMI >30-40, Grade 2 Overweight; BMI >40, Grade 3 Overweight), but do not present interaction p values because of the large number of comparisons and the exploratory nature of this analysis.

Sensitivity Analysis

To determine sensitivity of the results to outliers, we fit models to log-transformed adhesion proteins. This analysis was motivated by non-normal distributions of several adhesion proteins.

To determine whether there was a relationship between air pollution exposure and latent variables in the adhesion data, we performed a factor analysis on the adhesion proteins. Scaled adhesion molecules were used in a factor model. We specified 5 factor variables to have the same *a priori* number of variables as our pre-specified mechanistic categories of adhesion proteins (selectins, adhesion proteins, MMPs, chemokines, and growth factors); this allowed us to assess factor variable grouping (via the loadings) versus our pre-specified conceptual

categories. We then estimated the association between air pollution and each factor using separate statistical models.

3.3 RESULTS

Since adhesion markers were measures in a subset of participants at examination 2, we compare descriptive statistics between the baseline cohort (examination 1), the cohort at follow-up (examination 2), and the nested cohort selected at examination 2 for adhesion marker measurement (Table 3.2). We collected participant demographics at both baseline and examination 2, so the baseline and the time-varying variables are compared in this table. There were 6,814 participants recruited into MESA at examination 1, of which 6,233 participanted in examination 2 (Table 3.2). Subjects were randomized in equal proportions by race/ethnicity into the adhesion sub-cohort. There were 2,569 subjects at examination 2 for whom one or more adhesion measure is available. At examination 1, average participant age was 62.2 years. Average participant age at examination 2 was 63.6 years. The gender distribution of the cohort stayed relatively constant even as some subjects were missing at follow-up (53% women at examination 1 compared to 52% women at examination 2). There was a slight tendency for more educated individuals to participate in examination 2 (35% college or graduate at exam 1, 37% college or graduate at exam 2). At examination 2, approximately 46% of participants were never smokers, 43% were former smokers, and 11% were current smokers. Notably, there were 50% never smokers at baseline; this difference is largely attributable to differing in self-report of smoking status between examinations rather than being due to loss-to-follow-up. Of the participants who attended examination 2, approximately 38% were white, 12% were Chinese, 28% were black, and 22% were Hispanic.

Adhesion Related Proteins

Sample sizes and serum/plasma concentrations differed between the 15 adhesion proteins measured. Table 3.3 describes the distribution of protein concentrations and sample sizes. Figure 3.1 shows the distribution of each protein. Most proteins have some amount of right skew, although it is most notable in ICAM-1 and CCL5. Pearson correlations between proteins are presented in Table 3.4. CCL5 and TGFB-1 have a strong positive correlation value of 0.79. MMP-2 and TIMP-1 have a strong positive correlation value of 0.78. No other correlation values were greater than 0.5 or less than -0.5. Given the relatively low correlations between variables, the Bonferroni approach was used to adjust for multiple comparisons.

Exposures

Mean long-term $PM_{2.5}$ exposure in the year prior to examination 2 was 10.3 $\mu g/m^3$ with an interquartile range of 4.9 $\mu g/m^3$ (Table 3.5). The interquartile range for participants with one or more measured adhesion molecule was slightly larger at 6.1 $\mu g/m^3$. Mean NO_X long-term exposure over this period was 45 ppb with an interquartile ranges of 42.1 ppb. Because acute $PM_{2.5}$ exposure was preadjusted, the resulting preadjusted estimates are mean-centered by site over the complete time-series of monitoring data. In the subset of measurements corresponding to days prior to MESA examination 2, the mean was -0.1 $\mu g/m^3$ with an interquartile range of 8.2 $\mu g/m^3$.

Exposures differed greatly by study site. Long-term estimated $PM_{2.5}$ exposure was highest in Los Angeles and lowest in the Twin Cities (Figure 3.2). Estimated NO_X exposures were highest in New York and Los Angeles and lowest in Winston Salem and the Twin Cities

(Figure 3.3). Adjustment for seasonality, temperature, and relative humidity resulted in a more symmetric distribution of acute exposures (Figure 3.4).

Association between Air Pollution and Adhesion Markers

Figure 3.5 presents estimates of association corresponding to long-term $PM_{2.5}$ and NO_X exposure, scaled to the respective inter-quartile range of each adhesion molecule for the long-term for each combination of adjustment approach (Model 1, Model 2). One protein, CCL21, was significant at the Bonferroni level (adjusting for number of proteins times the number of long-term pollutants, α=0.05/30 or 0.0017) for both pollutants. Participants differing by 5 $\mu g/m^3$ of year-prior $PM_{2.5}$ exposure on average differed by 70.20 pg/mL of CCL1 (95% CI: 26.65, 113.74, p=0.0016) after adjusting for the variables in model 2. Participants differing by 42.1 ppb of year-prior NO_X exposure on average differed by 60.38 pg/mL of CCL21 (95% CI: 23.87, 96.89, p=0.0012) after adjusting for the variables in model 2. Model 2 adjusted estimates were 29.15 ng/mL of ICAM-1 (95% CI: 9.73, 48.57, p=0.0033) for 5 $\mu g/m^3$ $PM_{2.5}$ and 22.81 ng/mL of ICAM-1 (95% CI: 6.51, 39.11, p=0.0061) for 42.1 ppb NO_X. The model 2 adjusted estimate was 3.13 ng/mL of TIMP-2 (95% CI: 1.07, 5.19, p=0.0029) for 5 $\mu g/m^3$ $PM_{2.5.}$ While ICAM-1 and TIMP-2 did not meet strict Bonferroni significance adjustment, the sizes of the estimates of associations for these proteins were large and were estimated with greater precision than other results. The associations between these proteins and air pollution exposure was relatively strong given the overall distribution of the proteins in the cohort (Figure 3.5). Other associations corresponding to strong estimates of association but less precise confidence intervals were: VCAM-1 for both pollutants, CCL5 for $PM_{2.5}$ only, IL2-SR for $PM_{2.5}$ only, HGF for both pollutants, and MMP-2 for $PM_{2.5}$. As a sensitivity analysis we fit models to log-transformed

adhesion protein values. Exponentiated estimates from log models are presented in Figure 3.6. Log-scaled CCL21 was positively associated with year-prior $PM_{2.5}$ and NO_X. A 5 $\mu g/m^3$ interval in year-prior $PM_{2.5}$ exposure was associated with a 6% higher (95% CI: 1.01, 1.12, p=0.0116) geometric mean of CCL21 and a 42.1 ppb interval in NO_X was associated with a 6% higher (95% CI: 1.02, 1.10, p=0.0070) geometric mean of CCL21.

We fit interactions between long-term pollutant exposures and smoking status at examination 2 adjusting for the variables in model 2 (Figure 3.9). Associations were notably stronger in current smokers for CCL21, ICAM-1, VCAM-1, IL2-SR for $PM_{2.5}$, and HGF for both $PM_{2.5}$ and NO_X. Associations were largely similar in former and never smokers. We also present interaction results by BMI category (Figure 3.10). There was a stronger association of air pollution with increasing BMI categories for both pollutants in ICAM-1, MMP-2, and TIMP-2. The association of air pollution with CCL21 and HGF was constant and elevated in BMI categories with the exception a null effect in individuals classified as Grade 3 Overweight.

Acute $PM_{2.5}$ Exposure and Adhesion Proteins

Acute $PM_{2.5}$ exposure and adhesion protein associations are presented in Figure 3.7. No results were significant at the Bonferroni level (alpha=.05/15), and in general the estimated associations were small compared to each respective interquartile range of the proteins in MESA. Two proteins, ICAM-1 and SLP1, were associated with day-prior $PM_{2.5}$ exposure at standard significance (alpha=0.05). For participants differing by 5 $\mu g/m^3$ of day-prior $PM_{2.5}$ exposure on average differed by -4.22 ng/mL of ICAM-1 (95% CI: -7.14, -1.30, p=0.0047) controlling for the variables in Model 2. The estimated association between acute $PM_{2.5}$ and ICAM-1 was negative, in contrast with the positive association observed for long-term $PM_{2.5}$ exposure. For participants

differing by 5 μg/m^3 of day-prior $PM_{2.5}$ exposure on average differed by 299.29 pg/mL of SLP1 (95% CI: 17.19, 581.40, p=0.0376) controlling for the variables in Model 2. Log-scale results (Figure 3.8) were not appreciably different from the untransformed results.

We fit interactions between acute $PM_{2.5}$ exposure and smoking status adjusting for the variables in model 2 (Figure 3.11). Estimates of association for ICAM-1 were slightly stronger (more negative) in current smokers, but this difference was not large. There were no consistent results for interaction by smoking status and acute $PM_{2.5}$ exposure for SLP1. BMI-specific estimates were closer to the null in individuals classified as Grade 3 overweight for both ICAM-1 and SLP1, although confidence intervals for this group were much wider (Figure 3.12).

Factor Analysis

Approximately 48% of the variability in our sample of adhesion proteins is explained by three factor loadings, where the first through fifth factors described 12%, 11%, 11%, 9%, and 5% of the variability, respectively (Table 3.6). Loadings indicate the factor analysis grouped the two pairs of correlated variables together into two separate factors (Table 3.7). Factor 1 had strong loading values for CCL5 (factor loading 0.81) and TGFB-1 (factor loading 0.96). These two adhesion proteins have a Pearson's correlation value of 0.79. P-Selectin was the only other notable variable included in Factor 1 with a factor loading value of 0.39. Factor 2 loads heavily on MMP-2 (factor loading 0.99) and TIMP-1 (factor loading 0.75). These two adhesion proteins have a Pearson's correlation value of 0.78. Factor 3 loads strongly on CCL21 (0.57), HGF (0.57), and SLP1 (0.53) as well as less strongly on the three adhesion molecules (ICAM-1 0.30; E-cadherin 0.36; VCAM-1 0.25), P-selectin (0.36), and IL-2sr (0.41). Factor 4 loads heavily on VCAM-1 (0.70), as well as ICAM-1 (0.30), IL-2sr (0.44), and L-Selectin (0.46). Factor 5 loads

on SDF-1a (0.52), VCAM-1 (0.39), and CCL21 (0.39) with slight negative loading on P-Selectin (-0.23) and ICAM-1 (-0.14).

Associations between the factors and long-term air pollution exposures are presented in Figure 3.13. $PM_{2.5}$ was significantly negatively associated with factor 1, significantly positively associated with factors 2 and 3, and had suggestive positive associations with factors 4 and 5. NO_X had a significant positive association with factor 3 and suggestive positive associations with factors 4 and 5.

3.4 DISCUSSION

In a large multi-ethnic cohort of individuals free from cardiovascular disease at recruitment, we found that year-prior exposure to $PM_{2.5}$ and NO_X were both significantly positively associated with the chemokine CCL21, that both pollutants had strong positive associations with ICAM-1, and that $PM_{2.5}$ had a strong association with TIMP-2. The p-values for TIMP-2 and ICAM-1 did not meet the stringent Bonferroni significance cutoff but were strong in the size of association and these coefficients were estimated with relative precision. There were additional proteins which had strong associations that were significant according to the standard significance cutoff ($p < 0.05$) but not Bonferroni adjusted significance ($p < 0.05/30$): VCAM-1 for both pollutants, CCL5 for $PM_{2.5}$ only, IL2-SR for $PM_{2.5}$ only, HGF for both pollutants, MMP-2 for $PM_{2.5}$, TGFB-1 for $PM_{2.5}$. Given the number of comparisons, the results are most compelling for CCL21, ICAM-1, and TIMP-2. However, the overall number of non-null associations presents relatively strong statistical evidence that long-term exposure to these pollutants and $PM_{2.5}$ in particular may be cross-sectionally associated with adhesion processes. The acute $PM_{2.5}$ exposure analyses for the most part showed no evidence of an association. We

did find a negative association between $PM_{2.5}$ and ICAM-1 and a positive association between $PM_{2.5}$ and SLP1, but these associations were relatively weak and not estimated with sufficient precision to be compelling given the number of comparisons. The general conclusions of our results given by direction of association and approximate significance did not differ when we considered log transformed outcomes.

CCL21 had a strong association with both pollutants. The adjusted estimate of association for a 5 $\mu g/m^3$ interval in $PM_{2.5}$ was 70.20 pg/mL (95% CI: 26.65, 113.74, p=0.0016) and the adjusted effect estimate for a 42.1 ppb interval in NO_X was 60.38 pg/mL (95% CI: 23.87, 96.89, p=0.0012). The magnitude of these associations is relatively strong, equal to approximately 20% of the interquartile range of the distribution of CCL21 observed in our sample. CCL21 is a chemokine which binds to the CCR7 receptor and is primarily involved in T cell education and priming by guiding interactions between receptor presenting cells and T cells. CCL21 may interact with ICAM-1 to promote lymphocyte adhesion (Dominguez and Hammer, 2014). In an animal model, rats exposed to $PM_{2.5}$ had triple the relative CCL21 expression, although these findings were not variable and not significant (Oliveira-Fonoff et al., 2017); otherwise, this outcome has not been studied in relation to air pollution exposure. CCL21 is upregulated in atherosclerotic plaques in humans and $APOE^{-/-}$ mice which suggests that CCL21 may be implicated in abnormal T-cell recruitment to inflammatory sites and in extra-cellular matrix degradation via promotion of MMP levels(Damås et al., 2007). Most existing research on CCL21 is in response to allergens, particularly with regards to contact dermatitis. CCL21 is induced by a non-antigen specific response in the skin (Eberhard et al., 2004). CCL21 is also expressed in the lungs (Rangel-Moreno et al., 2007). Further research is needed to determine

whether air pollutants can act as an irritant to upregulate CCL21 in the lung, blood, or endothelium.

The strong positive relationship between tissue inhibitor of metalloproteinase 2 (TIMP-2) and year prior $PM_{2.5}$ exposure approached significance at the Bonferroni threshold. We additionally observed a positive relationship between MMP-2 and $PM_{2.5}$. These two proteins were strongly correlated in our dataset, which may be due to their functional relationship. TIMP-2 assists membrane type 1 MMP (MT1-MMP) in cleaving pro-MMP2 to make MMP2 (Sato and Takino, 2010). Since TIMP-2 is directly involved in regulating MMP2 the correlation between these two proteins and their similar association with $PM_{2.5}$ are unsurprising. A study in mice found MMP-2 expression in response to acute gasoline exhaust exposure to be modified by the supplementation of an anti-oxidant, suggesting reactive oxygen species as a mediator of this relationship (Lund et al., 2009). Other researchers have shown increases in TIMP-2 and MMP2 in rat lung epithelial and inflammatory cells following intratracheal instillation of particulate matter suggesting metalloproteinase response to inhaled pollution may also occur in lung tissue (Su et al., 2000).

$PM_{2.5}$ and NO_X pollutants were positively associated with ICAM-1 with estimates that correspond to approximately 25% of the ICAM-1 sample interquartile range for $PM_{2.5}$ and 30% for NO_X. Our results confirms epidemiologic evidence which identified significant positive associations with circulating ICAM-1 in humans in relation to $PM_{2.5}$ and NO_2 exposure (Bind et al., 2012). Human controlled exposure experiments also suggest a relationship between particulate exposure and ICAM-1; in one analysis circulating soluble ICAM-1 was elevated in response to concentrated ambient $PM_{2.5}$ exposure (Gong et al., 2003) while another analysis found no significant difference (effect size not reported) in soluble ICAM-1 concentration in

relation to diesel exhaust but did show increased ICAM-1 protein expression and ICAM-1 ligand protein expression in relation to diesel exhaust exposure (Salvi et al., 1999). Additional in vitro studies have also shown strong associations with ICAM-1 surface expression (doubling in normalized expression) in relation to diesel exhaust particle powder in epithelial cells (Takizawa et al., 2000) and $PM_{2.5}$ extract in endothelial cells (Montiel-Dávalos et al., 2007; Rui et al., 2016).

Given that the majority of existing research on the positive association between $PM_{2.5}$ and ICAM is in relation to acute exposure (controlled exposures or in vitro studies), it is surprising that our results show a strong positive association (in the expected direction of effect) for long-term exposure and a negative association (in the unexpected direction) for acute exposure. It is worth noting that most of these acute studies showed pollution-induced surface expression rather than effects on circulating ICAM-1. ICAM-1 shedding from cellular surfaces may involve ADAM-17 (tumor necrosis factor alpha converting enzyme /TACE) (Tsakadze et al., 2006), but the relationship between air pollution exposure and ICAM-1 shedding is unclear. Further research is needed to understand how $PM_{2.5}$ might specifically affect ICAM-1 transcription, surface expression, and shedding and which cell types (e.g., endothelial or pulmonary epithelial) could be involved in air pollution induced changes in circulating ICAM-1.

The clinical significance of ICAM-1 in cardiovascular pathobiology is unknown, although epidemiologic evidence suggests it may at least be a predictor of cardiovascular events in humans. Some but not all experimental studies in mice suggest that ICAM-1 may be a causative agent in atherosclerosis (Collins et al., 2000; Manka et al., 2001), but whether or not ICAM-1 is a causative agent, it appears to be a strong biomarker of coronary risk independent of conventional risk factors in humans. ICAM-1 is associated with coronary events: Several nested-

case control analyses within prospective cohorts show circulating ICAM-1 is associated with a large increase in risk of CHD events, independent of traditional risk factors. For example, ICAM-1 was associated with a relative risk of 2.09 (95% confidence interval: 1.34-3.24 3rd quartile of greater than 625 ng/ml versus 1st quartile of less than 502 ng/ml) for coronary events after adjusting for age, diabetes, hypertension, smoking, LDL, HDL, and CRP during a 5-year follow-up in a study in men aged 50-59 (Luc et al., 2003). The underlying mechanisms which result in ICAM-1 elevation in response to air pollution exposure remain unclear, although evidence suggests that this relationship may be mediated by oxidative stress. NO is degraded by oxidative stress, and inhibition of endothelial-mediated NO production may increase expression of ICAM-1 expression (Lindemann et al., 2000). In vitro $PM_{2.5}$ exposure was shown to increase ICAM-1 surface expression as a result of in changes in intracellular oxidative stress (Rui et al., 2016). Further research is needed to determine the mechanisms through which air pollution alters ICAM-1 expression.

Factor Analysis Results

Our factor analysis provided additional insight into the interrelationship between the adhesion proteins and the exposure estimates. Notably, the first two factors correspond to two pairs of correlated variables: CCL5 and TGFB-1 for factor 1 and MMP-2 and TIMP-1 for factor 2. The relationship between MMP2- and TIMP-1 has been well-characterized and is described above. CCL5 and TGFB-1 both were negatively associated with $PM_{2.5}$. Results for these variables were consistent with both a strong effect as well as no effect given uncertainty in the estimates of association due to multiple comparisons; but do have suggestive negative associations with $PM_{2.5}$, as does P-selectin which is also loaded strongly in factor 1. TGFB-1 is

involved in down-regulating the inflammatory chemokine CCL5 (Cho et al., 2006). In our study these proteins were correlated, indicating a tight level of regulation. TGFB-1 plays a complex role in inflammation and may not be uniquely pro-inflammatory or anti-inflammatory. In a rat model involving inhibited NO synthesis, TGFB-1 is released in monocytes infiltrating into vessel lesions and treatment with TGFB-1 antibodies inhibits inflammation and monocyte infiltration as well as inhibiting expression of P-selectin and ICAM-1 (Koyanagi et al., 2000). This study indicates that TGFB-1 may be involved in inflammatory changes that promote P-selectin expression, although it is not clear why in our analysis $PM_{2.5}$ was negatively associated with these markers. Previous researchers have established a positive relationship between $PM_{2.5}$ and TGFB-1 in a study of rats (Oliveira-Fonoff et al., 2017). The authors suggest this relationship may be mediated by oxidative stress and could explain air pollution induced myocardial remodeling.

Interpreting the results of the factor analysis demonstrates the limitation of single-protein analyses and our *a priori* mechanistic categories. Some proteins in different mechanistic categories, such as CCL5 and TGFB-1 were correlated and loaded on the factors resulting in factors that were associated with the outcome, although the strength of the estimates for the factors is difficult to interpret meaningfully. In this way, factor analysis was able to capture relationships amongst strongly correlated adhesion molecules and allowed us to identify data driven associations that were agnostic of our understanding of the underlying mechanisms. This factor analysis facilitates a systems approach to interpreting relationships between air pollutants and correlated adhesion proteins. There are many different pathways involved in these adhesion proteins, and although this research approach is not capable of definitively identifying individual

pathways in the pathophysiology of air pollution exposure, our approach may suggest individual proteins or pathways for future study.

Reactive Oxygen Species and Nuclear Factor Kappa Beta

$PM_{2.5}$ induces reactive oxygen species which upregulate the transcription factor nuclear factor kappa beta (NF-kB) which may be responsible for $PM_{2.5}$-induced ICAM-1 surface expression (Rui et al., 2016), although it is unclear whether air pollution induced intracellular changes in oxidative stress results directly from endogenous ROS molecules in air pollution or whether air pollution induces inflammatory cascades which promote endogenous ROS. Many of the proteins positively associated with $PM_{2.5}$ exposure in our analyses may be regulated in part by nuclear factor kappa beta (NF-kB). For example, a non-canonical NF-kB pathway regulates CCL21 expression (Valiño-Rivas et al., 2016), and NF-kB also has bindings sites on the promoter region of the gene that encodes CCR7, the receptor for CCL21 (Höpken et al., 2002).

Conversely, SDF1-a was not associated with $PM_{2.5}$ exposure in our results, even though NF-kB is involved in SDF1-a transcription. However, there is cross-talk between canonical and non-canonical NF-kB in SDF1-a regulation (Madge and May, 2010), which could potentially explain the absence of an association with $PM_{2.5}$ even if $PM_{2.5}$ does initiate an NF-kB signaling cascade. NF-kB also regulates the chemokine CCL5 (Moriuchi et al., 1997; Valiño-Rivas et al., 2016), and we found a negative association between $PM_{2.5}$ and CCL5. We additionally found a negative association between TGFB-1 and $PM_{2.5}$. TGFB-1 may regulate CCL5 through NF-kB (Cho et al., 2006). NF-kB is also involved in regulating ICAM-1 (Sun et al., 2001) and matrix metalloproteinases (Wu and Schmid-Schönbein, 2011). Conversely, TIMP-2 may promote cell growth by upregulating NF-kB (Lizárraga et al., 2004). While many of the proteins we observed

to be associated with $PM_{2.5}$ are related to NF-kB, different mechanisms have been found to activate the NF-kB pathway for different proteins. Notably some of the proteins are upstream, rather than downstream, of NF-kB, and the direction of effect between NF-kB, individual proteins, and their associations with $PM_{2.5}$ is complex. Therefore, if $PM_{2.5}$ does alter adhesion proteins in humans, it is due to a complex set of relationships rather than due to a straightforward upregulation of a single transcription factor. Furthermore, without measuring gene expression, we are unable to determine whether activation of this transcription factor could be directly involved in the relationships observed in this analysis. However, our results suggest further research into the transcriptional regulatory networks connecting air pollution exposure and adhesion proteins is necessary.

Effect Modification

Our ability to draw conclusions about possible effect modification are limited due to our study size, which was not powered to analyze effect modifications especially considering the number of comparisons in the primary analysis. We did not formally assess interaction significance for these analyses because the interaction models were exploratory. However, there were a few notable trends that may warrant external validation. The associations of long-term $PM_{2.5}$ and NO_X exposure with CCL21 and HGF were both stronger in current smokers. This result is somewhat surprising given because it suggests potentiation, rather than attenuation between the relatively similar exposures of $PM_{2.5}$ and smoking. Substantial research exists showing an association between smoking status and HGF, but further pathway research is needed to determine how this relationship may interact with $PM_{2.5}$ exposure. We also observed different associations for individuals classified as grade 3 overweight. However, given the uncertainty of

these estimates relative to the other categories few conclusions can be reliably drawn from this result.

Strengths and Limitations

Our study is limited by a number of features. Although our original hypothesis was motivated by interest in air pollution, cardiovascular disease, and endothelial adhesion, we are unable to uniquely identify effects to the endothelium given the design of this study. All adhesion-related markers were measured in either plasma or serum and therefore may not reflect the expression of these molecules at endothelial vascular beds or in any specific organ or cell type. Air pollution induced adhesion expression can occur in pulmonary tissue as well as vascular endothelial cells. For example, elevated ICAM-1 expression in human bronchial epithelial cells is associated with response to diesel exposure (Takizawa et al., 2000), but elevated ICAM-1 expression in response to $PM_{2.5}$ has also been observed in endothelial cells (Montiel-Dávalos et al., 2007; Rui et al., 2016). However, even if air pollution alters surface expression of adhesion molecules exclusively in pulmonary epithelial tissue, air pollution might concurrently induce these cells to express other circulating inflammatory signaling molecules that could result in downstream endothelial changes. The limitation of using circulating proteins inhibits our ability to make conclusions about the specific site of adhesion molecule expression, and further mechanistic research would be needed to determine whether endothelial cells specifically are expressing these markers.

Another potential limitation of our study is multiple comparisons. However, we used a strict Bonferroni correction to guide the interpretation of our results. The multiple comparison

adjustment prevents overemphasis of results which are consistent with both strong effects and no effect. The only significant result robust to Bonferroni adjustment was CCL21. Associations relating $PM_{2.5}$ with ICAM-1 and TIMP-2 were strong given the overall distribution of these variables in MESA. While these results had p-values slightly larger than the Bonferroni cutoff of $p < 0.05/30$, we nevertheless place special emphasis on these variables because the precision in estimation of these variables sets them apart from other results. Other results were consistent with large effects and therefore potentially interesting but due to the large number of comparisons and the hypothesis generating nature of this analysis we do not promote strong conclusions regarding these other proteins. Further research might confirm other findings but our analysis was limited in power due to sample size and number of comparisons.

This analysis was purely cross-sectional which precludes our ability to make statements about whether change over time in circulating molecules is related to air pollution exposure. This design limits our confidence that the observed effects are causal, although many of our results do replicate effects in animal experimental models for pollutant exposures on various proteins. Nevertheless, to further validate our results and previous experimental evidence, more epidemiologic research is required. A first step would involve simple replication of these results, due to the exploratory nature of this analysis, using another population and a larger sample. More involved study designs involving repeated measures and longitudinal analysis may further strengthen the case for a physiologic relationship between air pollution exposure in humans and adhesion processes. Additionally, we cannot rule out confounding by unmeasured factors. However, our results were reassuringly insensitive to the adjustment model we used.

Our analysis was also limited by using single-site monitors to estimate acute exposure. Evidence for an association between long-term exposure and various proteins was much more

compelling than evidence for an association with acute exposure. Given the different exposure classification approaches between acute and long-term exposure, it is not possible to conclude whether this difference in long-term and short-term associations is due to a biological effect relating to time-scale of exposure or simply because there may be more measurement error in the acute estimates. However, previous studies using this approach for estimating acute $PM_{2.5}$ exposure have found associations with biological measures in humans, suggesting the limitation may not be in the exposure assessment (Adar et al., 2010). Due to the difficulty in directly answering this question of time-scales of exposure, limited research exists on the duration of air pollution needed to induce and latency period involved in change in human inflammatory biomarkers. There is limited and conflicting evidence in controlled exposure studies regarding the effect of air pollution on adhesion makers. One study showed small and nonsignificant increases in circulating ICAM-1 and VCAM-1 as a result of diesel exhaust exposure compared to filtered air (Krishnan et al., 2013). On the other hand, considerable in-vitro evidence also links $PM_{2.5}$ exposure to relatively large changes ICAM expression (Ishii et al., 2005; Montiel-Dávalos et al., 2007). It is possible that future carefully designed epidemiologic analyses involving serial measurement of proteins over extended periods could help answer this question.

Since oxides of nitrogen are spatially heterogeneous, we were not able to use the single – site monitor approach to estimate acute NO_X exposures. Additionally, due to the multiple comparisons issue, we did not have the power to assess multiple periods of exposure for acute $PM_{2.5}$. We instead chose to use a single biologically plausible period, day prior, for these biomarkers. Sensitivity analyses (not shown) show that the estimates of association are not sensitive to altering the lag period or averaging periods by a few days.

Strengths of this study include a cohort with high-quality health measures in individuals free of cardiovascular disease at baseline, well-validated year prior annual exposure estimates, and well-characterized confounder information on variables such as smoking, BMI, and other cardiovascular risk factors.

In conclusion, we found associations between long-term pollutants and several adhesion molecules. NO_X exposure was strongly associated with ICAM-1 and CCL21. $PM_{2.5}$ was strongly associated with CCL21, ICAM-1, and TIMP-2 and had suggestive associations with several other proteins. The strengths of the associations between acute $PM_{2.5}$ exposure and adhesion proteins were generally weak or null. While the design of this study does not allow conclusive elucidation of biological pathways, the results suggest that long-term air pollution exposure may involve adhesion processes, inflammation, and possibly pathways involving reactive oxygen species and transcription factor NF-κB. Additional research into the biologic mechanisms of these molecules is necessary to better understand whether these associations may mediate the effect of air pollution on cardiovascular disease.

3.5 FIGURES

Figure 3.1. Distributions of Adhesion Related Proteins

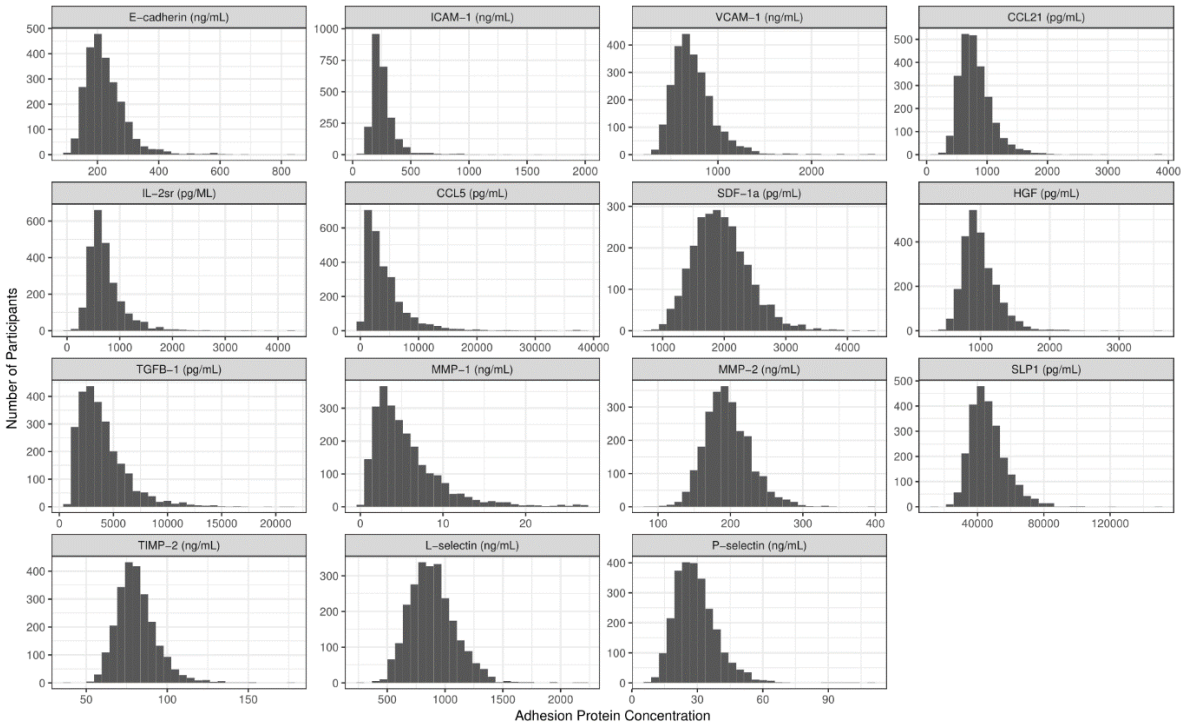
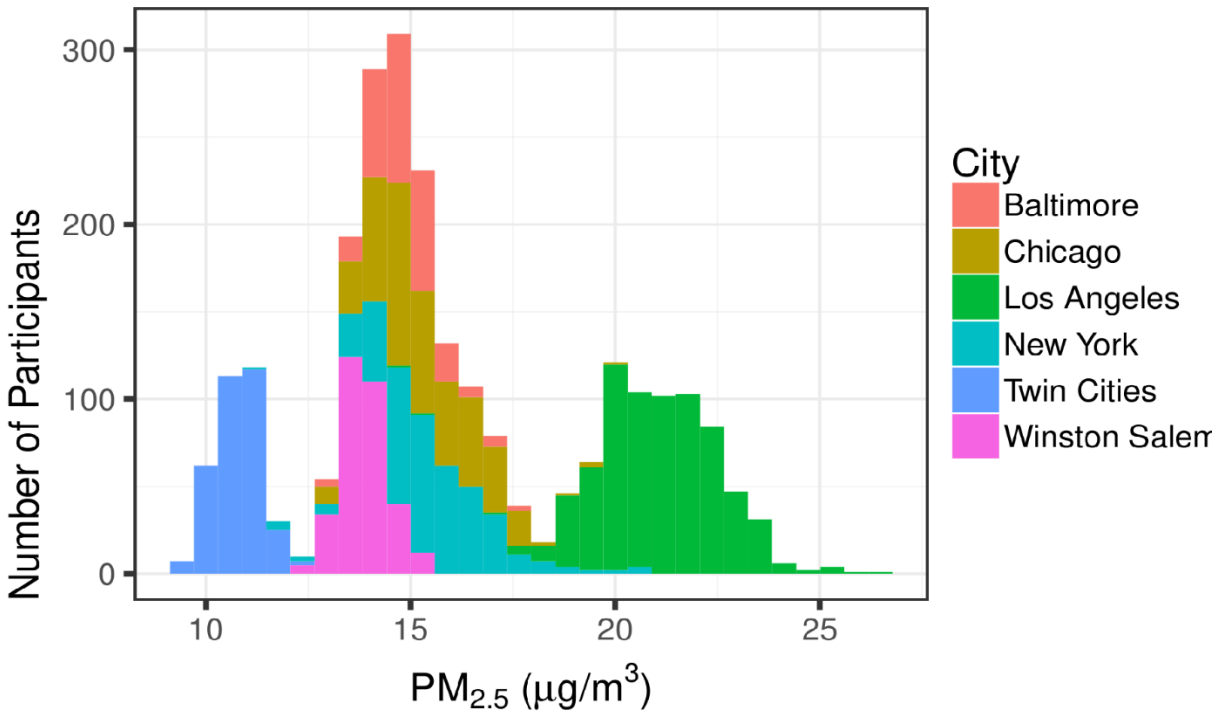
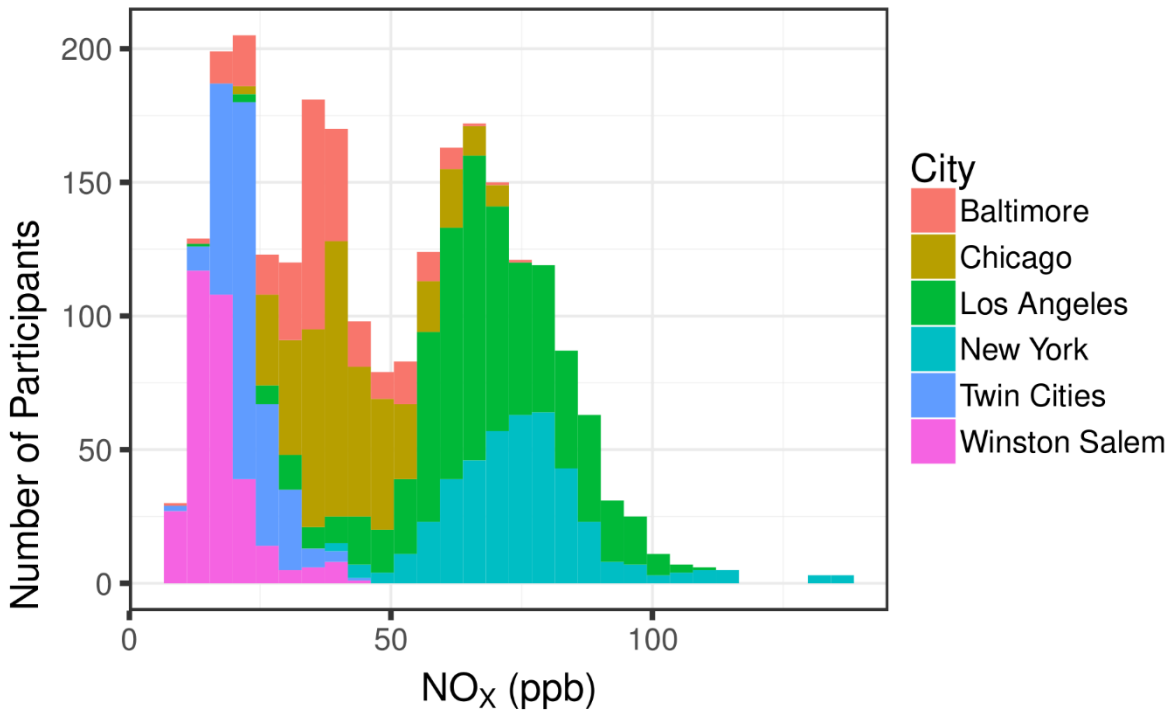


Figure 3.2. Distribution of Long-term $PM_{2.5}$ Exposure by Study Site



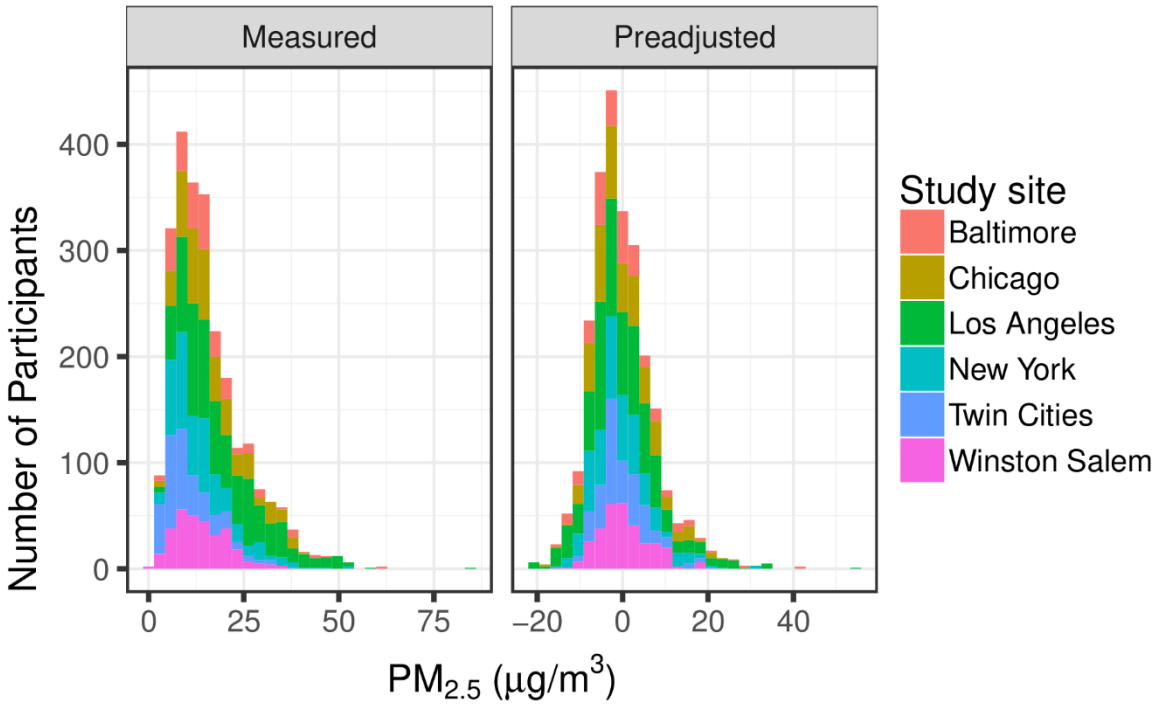
For exposure estimated over the year prior to examination 2 in all MESA participants who had any measured adhesion molecule at examination 2.

Figure 3.3. Distribution of Long-term NO_X Exposure by Study Site



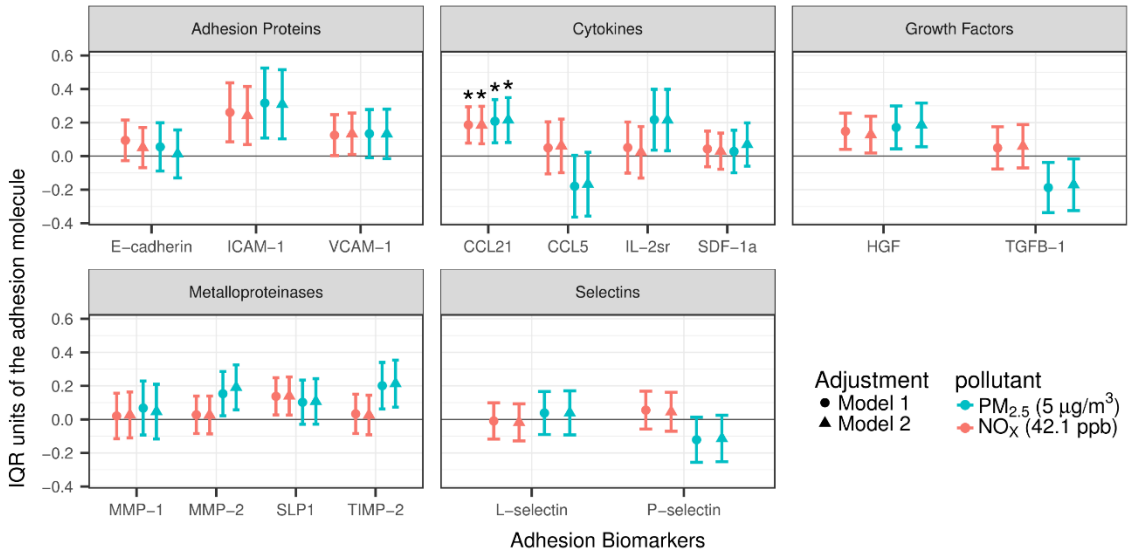
For exposure estimated over the year prior to examination 2 in all MESA participants who had any measured adhesion molecule at examination 2.

Figure 3.4. Distribution of Acute $PM_{2.5}$ Exposure: Measured and Pre-adjusted Day-Prior Exposure



Measured and preadjusted site-specific exposure values from central monitors on day of examination 2. Values were preadjusted with seperate models by site, resulting in mean centered values. This dataset is subsetted to individuals who had one or more adhesion proteins measured at examination 2.

Figure 3.5. Estimated Association between Long-term Air Pollution Exposure and the Adhesion-Related Proteins



Confidence intervals are standard (non-adjusted) and asterisks indicate Bonferroni adjusted significance

Figure 3.6. Estimated Association between Long-term Air Pollution Exposure and Log Adhesion-Related Proteins, Exponentiated Coefficients

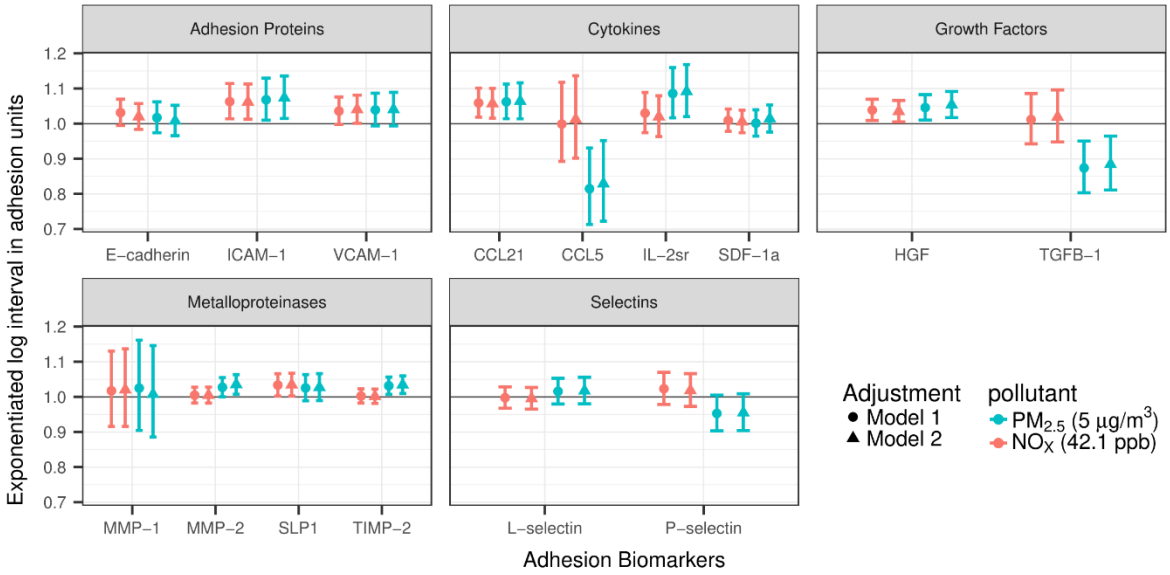


Figure 3.7. Estimated Association between Acute $PM_{2.5}$ Exposure and the Adhesion-Related Proteins

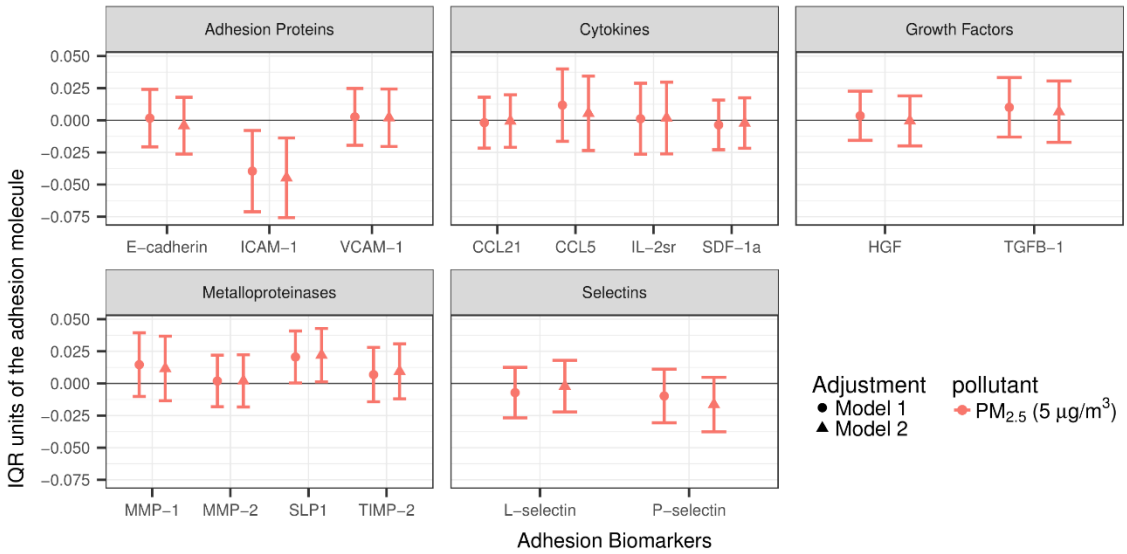


Figure 3.8. Estimated Association between Acute $PM_{2.5}$ Exposure and Log Adhesion-Related Proteins, Exponentiated Coefficients

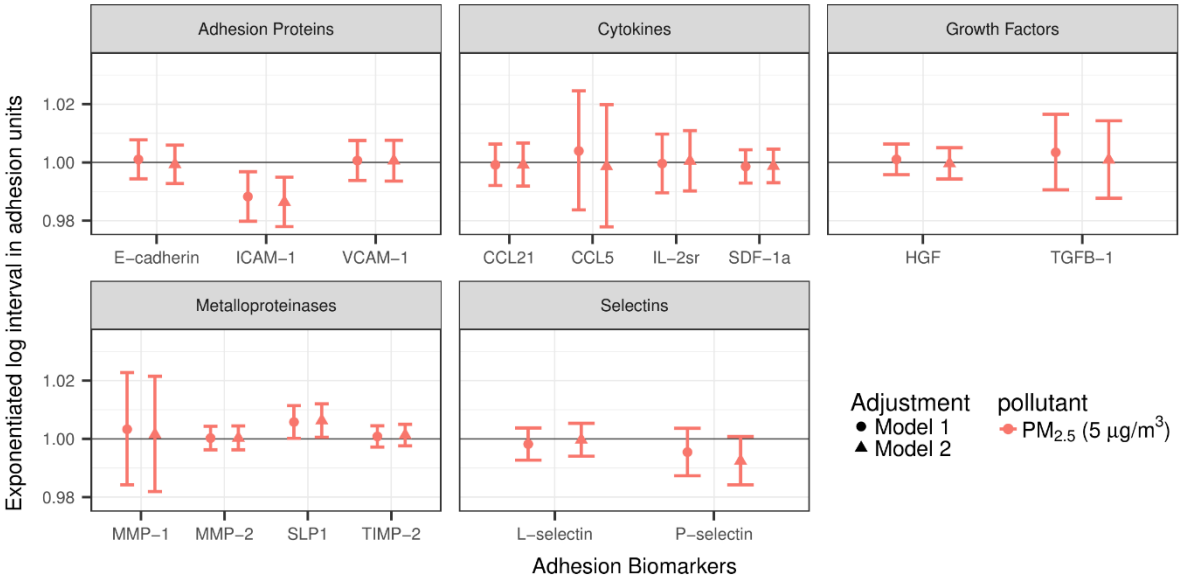


Figure 3.9. Interaction between Smoking Status and Long-term Air Pollution Exposure on the Adhesion-Related Proteins

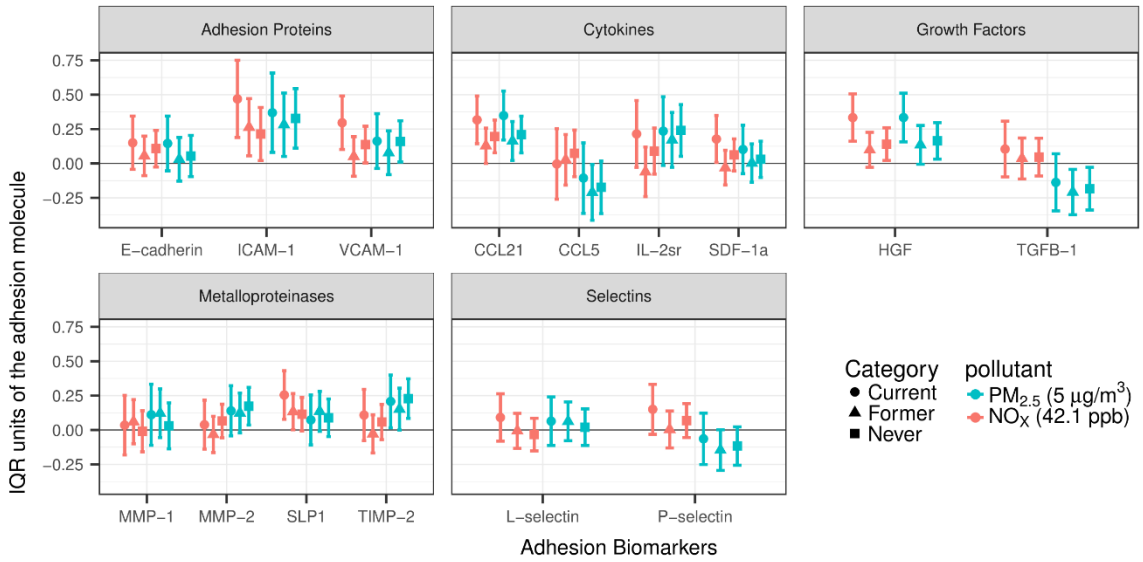


Figure 3.10. Interaction between BMI and Long-term Air Pollution Exposure on the Adhesion-Related Proteins

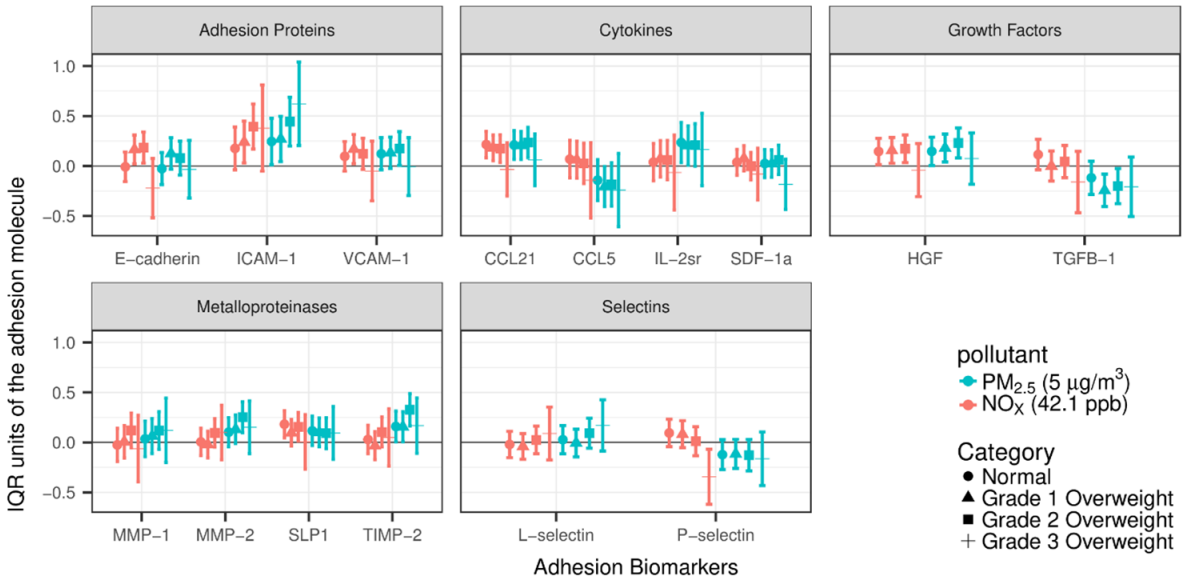


Figure 3.11. Interaction between Smoking Status and Acute $PM_{2.5}$ Exposure on the Adhesion-Related Proteins

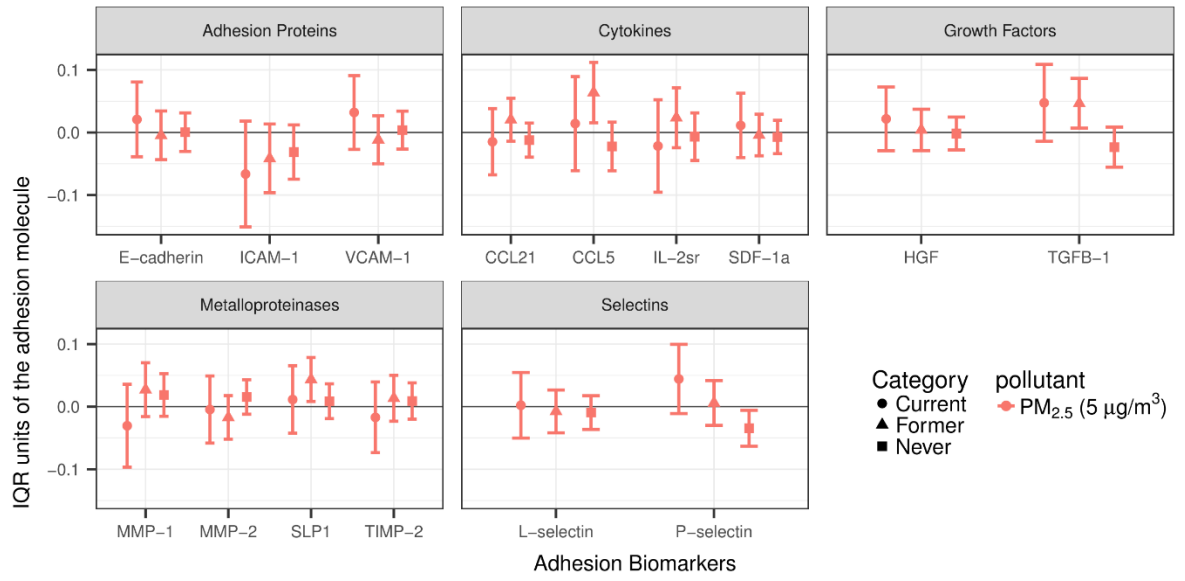


Figure 3.12. Interaction between BMI and Acute PM2.5 Exposure on the Adhesion-Related Proteins

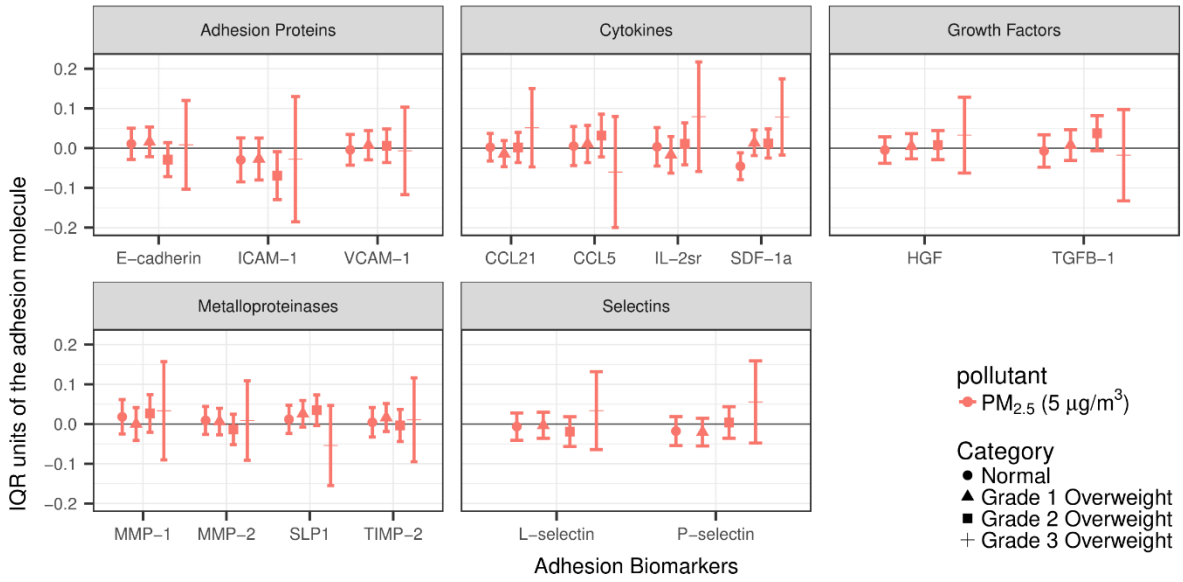
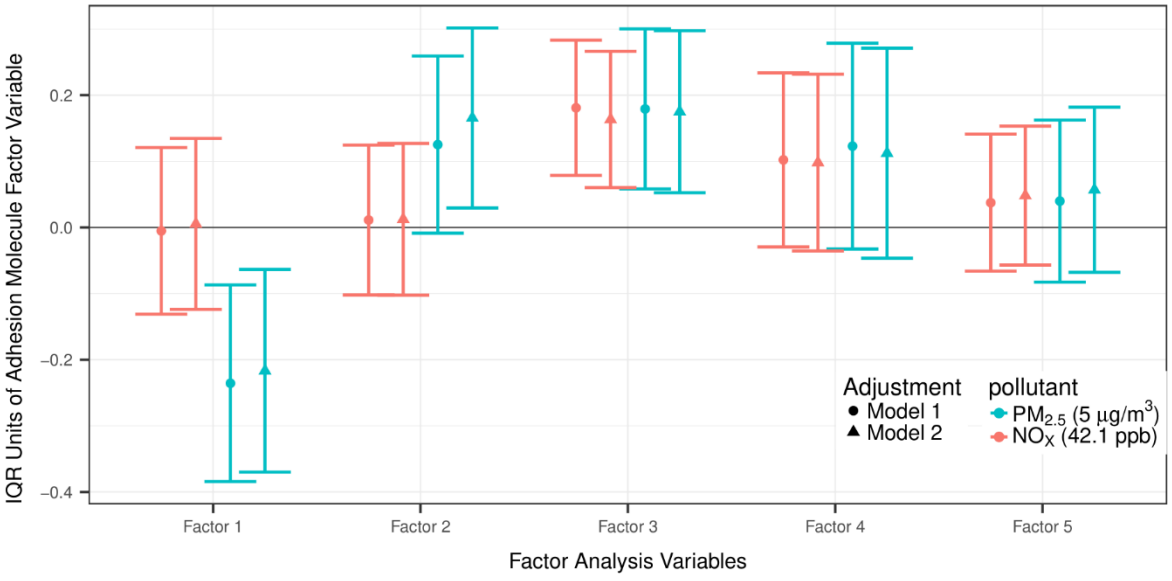


Figure 3.13. Estimated Association between Long-term Air Pollution Exposure and the Factors Created from Adhesion-Related Proteins



3.6 TABLES

Table 3.1 Adhesion Proteins, Assays, and Validity Statistics

Protein (units)	ELISA	Minimal detectable level	Inter-assay coefficient of variation (%)
Selectins: leukocytes and platelet adhesion to the endothelium			
L-selectin (ng/mL)	Human soluble L-selectin/CD62L Immunoassay kit (R&D Systems, Minneapolis, MN).	0.3 ng/mL.	6.7% at a mean concentration of 943 ng/mL (serum pooled control)
P-selectin (ng/mL)	Human soluble P-Selectin/CD62P Immunoassay kit (R&D Systems, Minneapolis, MN).	0.5 ng/mL	6.7% at a mean concentration of 182.1 ng/mL (Exam 2)
Inter and intra-cellular adhesion proteins			
E-cadherin (ng/mL)	Human E-Cadherin Quantikine ELISA Kit (R&D Systems, Minneapolis, MN)	0.039 ng/mL	7.8% at a mean concentration of 197 ng/mL
ICAM-1 (ng/mL)	Human sICAM-1 Instant ELISA (Bender MedSystems GmbH, Vienna, Austria)	2.17 ng/mL	9.1% at a mean concentration of 261 ng/mL
VCAM-1 (ng/mL)	Human sVCAM-1/CD106 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN)	0.6 ng/mL	3.6% at a mean concentration of 564 ng/mL
Matrix metalloproteinases and protease inhibitors: plaque remodeling, cell mobilization, inflammatory markers cleavage			
MMP-1 (ng/mL)	Human Pro-MMP-1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN)	0.021 ng/mL	3.5% at a mean concentration of 6.3 ng/mL
MMP-2 (ng/mL)	Total MMP-2 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN)	0.047 ng/mL	3.8% at a mean concentration of 186 ng/mL
SLPI (pg/mL)	Human SLPI Quantikine ELISA Kit (R&D Systems, Minneapolis, MN)	25 pg/mL	8.9% at a mean concentration of 36888 pg/mL
TIMP-2 (ng/mL)	Human TIMP-2 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN)	0.011 ng/mL	4.7% at a mean concentration of 73.3 ng/mL
Chemokines and cytokine receptor: inflammatory cells homing to lymph nodes or inflammation sites			
6Ckine/CCL21 (pg/mL)	Human CCL21/6Ckine Quantikine ELISA (R&D Systems, Minneapolis, MN)	9.9 pg/mL	5.6% at a mean concentration of 493 pg/mL
IL-2sr (ng/mL)	Human IL-2 sR alpha Quantikine ELISA (R&D Systems, Minneapolis, MN)	10 pg/mL	7.7% at a mean concentration of 653 ng/mL
RANTES/CCL5 (pg/mL)	Human CCL5/RANTES Quantikine ELISA Kit (R&D Systems, Minneapolis, MN)	2.0 pg/mL	10.0% at a mean concentration of 63287 pg/mL
SDF-1α (pg/mL)	Human CXCL12/SDF-1 alpha Quantikine ELISA Kit (R&D Systems, Minneapolis, MN)	18 pg/mL	11.4% at a mean concentration of 2228 pg/mL
Growth factors: cellular growth and proliferation			
HGF (pg/mL)	Human HGF Quantikine ELISA Kit (R&D Systems, Minneapolis, MN)	40 pg/mL	6.2% at a mean concentration of 946 pg/mL (Exam 2)
TGFβ-1 (pg/mL)	Human TGF-beta 1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN)	4.61 pg/mL	9.1% at a mean concentration of 32223 pg/mL

Courtesy of Susan Bielinski and Paul Decker, MESA Adhesion Study.

Table 3.2 Distribution of Participant Characteristics by Selection into Study

	Exam 1	Exam 2 Follow-up	Exam 2 Adhesion Cohort
N	6814	6233	2569
Age at baseline, y	62.2 (10.2)	62 (10.2)	61.3 (10.1)
Age at time of exam, y	62.2 (10.2)	63.6 (10.1)	63 (10)
Gender			
Male	3213 (47%)	2969 (48%)	1211 (47%)
Female	3601 (53%)	3264 (52%)	1358 (53%)
Race/ethnicity			
White	2622 (38%)	2464 (40%)	666 (26%)
Chinese	803 (12%)	728 (12%)	646 (25%)
Black	1893 (28%)	1691 (27%)	622 (24%)
Hispanic	1496 (22%)	1350 (22%)	635 (25%)
Education			
Less than high school	1225 (18%)	1044 (17%)	526 (21%)
High school	1236 (18%)	1124 (18%)	466 (18%)
Some college/technical	1937 (29%)	1773 (29%)	708 (28%)
College or graduate	2393 (35%)	2274 (37%)	862 (34%)
BMI at baseline, kg/m^2	28.3 (5.5)	28.3 (5.4)	27.9 (5.5)
BMI at time of exam, kg/m^2	28.3 (5.5)	28.4 (5.5)	28 (5.5)
Smoking at baseline			
Never	3418 (50%)	3142 (51%)	1404 (55%)
Former	2487 (37%)	2293 (37%)	816 (32%)
Current	887 (13%)	781 (13%)	342 (13%)
Smoking at time of exam			
Never	3418 (50%)	2857 (46%)	1282 (50%)
Former	2487 (37%)	2637 (43%)	970 (38%)
Current	887 (13%)	695 (11%)	295 (12%)
Diabetes at baseline			
Normal	4992 (74%)	4613 (74%)	1856 (72%)
Impaired fasting glucose	939 (14%)	855 (14%)	386 (15%)
Untreated diabetes	179 (03%)	158 (03%)	70 (03%)
Treated diabetes	680 (10%)	587 (09%)	253 (10%)
Diabetes at time of exam			
Normal	4992 (74%)	4225 (68%)	1724 (67%)
Impaired fasting glucose	939 (14%)	1052 (17%)	442 (17%)
Untreated diabetes	179 (03%)	166 (03%)	73 (03%)
Treated diabetes	680 (10%)	745 (12%)	328 (13%)

Summary values are mean (SD) or count (percentage).

Table 3.3 Distributions of Adhesion Related Proteins

Group	Adhesion Molecule	n	25th %ile	Mean	Median	75th %ile	IQR
Adhesion Proteins	E-cadherin (ng/mL)	2434	180.5	225.2	212.9	256.8	76.2
Adhesion Proteins	ICAM-1 (ng/mL)	2431	195.9	259.9	234.4	290.2	94.3
Adhesion Proteins	VCAM-1 (ng/mL)	2434	588.5	740.2	700.7	847.0	258.5
Cytokines	CCL21 (pg/mL)	2432	622.0	814.1	763.4	948.4	326.3
Cytokines	IL-2sr (pg/ML)	2432	514.5	731.5	647.3	840.1	325.6
Cytokines	CCL5 (pg/mL)	2561	1749.0	4300.3	3152.1	5462.5	3713.5
Cytokines	SDF-1a (pg/mL)	2566	1631.6	1948.7	1914.1	2212.5	580.9
Growth Factors	HGF (pg/mL)	2433	823.1	993.1	947.9	1123.8	300.6
Growth Factors	TGFB-1 (pg/mL)	2564	2374.1	3966.6	3451.2	4874.3	2500.2
Metalloproteinases	MMP-1 (ng/mL)	2434	2.7	5.6	4.5	7.2	4.5
Metalloproteinases	MMP-2 (ng/mL)	2434	174.6	196.0	193.1	214.6	39.9
Metalloproteinases	SLP1 (pg/mL)	2427	38670.1	46471.8	44761.0	52240.7	13570.6
Metalloproteinases	TIMP-2 (ng/mL)	2434	72.9	81.2	79.5	87.6	14.7
Selectins	L-selectin (ng/mL)	2434	753.6	890.8	874.5	1007.9	254.4
Selectins	P-selectin (ng/mL)	2566	22.5	29.3	27.9	34.4	11.9

Table 3.4 Pearson Correlation Matrix of Adhesion Related Proteins

	E-cadherin	ICAM-1	VCAM-1	CCL21	IL-2sr	CCL5	SDF-1a	HGF	TGFB-1	MMP-1	MMP-2	SLP1	TIMP-2	L-selectin
E-cadherin	1.00													
ICAM-1	0.21	1.00												
VCAM-1	0.36	0.37	1.00											
CCL21	0.24	0.16	0.37	1.00										
IL-2sr	0.29	0.30	0.50	0.39	1.00									
CCL5	0.03	0.06	-0.01	0.02	0.04	1.00								
SDF-1a	0.10	0.06	0.42	0.33	0.21	0.00	1.00							
HGF	0.27	0.37	0.36	0.37	0.33	0.12	0.22	1.00						
TGFB-1	0.06	0.03	-0.01	0.05	0.03	0.79	0.01	0.12	1.00					
MMP-1	0.02	0.01	-0.01	0.11	0.07	0.10	0.03	0.12	0.12	1.00				
MMP-2	0.18	0.08	0.29	0.22	0.12	-0.04	0.27	0.19	-0.06	0.02	1.00			
SLP1	0.29	0.21	0.26	0.37	0.29	0.08	0.22	0.32	0.08	0.06	0.15	1.00		
TIMP-2	0.20	0.12	0.34	0.29	0.21	-0.03	0.28	0.31	-0.04	0.05	0.78	0.19	1.00	
L-selectin	0.02	0.24	0.31	0.04	0.25	-0.03	0.08	0.11	-0.01	-0.05	0.01	-0.02	0.08	1.00
P-selectin	0.24	0.21	0.16	0.11	0.20	0.34	-0.03	0.32	0.39	0.09	0.01	0.19	0.01	0.03

Table 3.5 Distribution of Estimated Exposures for MESA Participants at Examination 2

Pollutant	units	Mean	25th %ile	75th %ile	IQR
$PM_{2.5}$, year prior	$\mu g/m^3$	10.3	7.3	12.2	4.9
NO_X, year prior	*ppb*	45.0	23.0	65.2	42.1
$PM_{2.5}$, preadjusted, day prior	$\mu g/m^3$	-0.1	-4.8	3.5	8.2

Table 3.6 Variability in the Adhesion Proteins Explained by Factors

	Factor1	Factor2	Factor3	Factor4	Factor5
SS loadings	1.76	1.70	1.64	1.40	0.75
Proportion Var	0.12	0.11	0.11	0.09	0.05
Cumulative Var	0.12	0.23	0.34	0.43	0.48

Table 3.7 Loadings from a Factor Analysis of the Adhesion Proteins

	Factor1	Factor2	Factor3	Factor4	Factor5
E-cadherin		0.14	0.36	0.28	
ICAM-1			0.30	0.49	-0.14
VCAM-1		0.21	0.25	0.70	0.39
CCL21		0.14	0.57		0.39
IL-2sr			0.41	0.44	0.19
CCL5	0.81				
SDF-1a		0.20	0.18	0.16	0.52
HGF		0.14	0.57	0.28	
TGFB-1	0.96				
MMP-1	0.10		0.19		
MMP-2		0.99			
SLP1			0.53		0.14
TIMP-2		0.75	0.20	0.11	0.16
L-selectin				0.46	
P-selectin	0.39		0.36	0.22	-0.23

Loadings with absolute values less than 0.10 are represented as blank

Chapter 4. MEDIATION OF THE RELATIONSHIP BETWEEN AIR POLLUTION AND CORONARY ARTERY CALCIUM

4.1 INTRODUCTION

Chronic ambient air pollution exposure is associated with subclinical cardiovascular disease and cardiovascular events, but the biological pathways for these relationships are not well understood. Atherosclerosis is a major component of cardiovascular disease, and air pollution exposure is associated with various measures of arterial injury including carotid intima media thickness (Bauer et al., 2010; Künzli et al., 2005; Perez et al., 2015) which reflects vessel wall thickening. In the Multi-Ethnic Study of Atherosclerosis (MESA), chronic $PM_{2.5}$ and NO_X exposure was associated with progression of coronary artery calcium (Kaufman et al., 2016) which is a clinical marker of plaque development and atherosclerosis.

Several biologic mechanisms have been proposed that could explain the relationship between air pollution and atherosclerosis. Acute controlled exposure studies suggest that air pollution may cause vascular changes such as alterations in blood pressure and endothelial function (Cosselman et al., 2012; Törnqvist et al., 2007). Several epidemiologic studies show associations between chronic air pollution exposure and blood pressure (Brook and Rajagopalan, 2009). In MESA, we previously observed suggestive associations between ambient air pollution and retinal measures of microvascular change, as well as cross-sectional associations between ambient air pollution and circulating biomarkers of cellular adhesion—a process which may be implicated in atherosclerotic lesion development. However, the extent to which these observed

vascular associations represent the causal pathway between chronic air pollution exposure and cardiovascular disease is unknown.

Assessing causal pathways using epidemiologic mediation is a potentially valuable tool that may strengthen the relevance of results involving the association between an exposure and a subclinical measure. We therefore analyzed the statistical mediation effect of central retinal arteriolar diameters (measured longitudinally) and three adhesion proteins (measured cross-sectionally) (CCL21, intercellular adhesion molecules 1 (ICAM-1), and tissue inhibitor of metalloproteinase 2 (TIMP-2)) with respect to the relationship between $PM_{2.5}$ and NO_X exposure and coronary calcification, a clinical measure of atherosclerosis.

4.2 METHODS

Study Population

Individuals aged 45 to 84 years old and free of cardiovascular disease were recruited as participants in a research study, the Multi-Ethnic Study of Atherosclerosis (MESA), in order to measure subclinical markers and clinical progression of cardiovascular disease. Recruitment of 6,814 participants occurred over the period between July 2000 to August 2002 from six metropolitan areas in the United States (Los Angeles, California; Minneapolis-St. Paul "Twin Cities", Minnesota; Chicago, Illinois; Baltimore, Maryland; Winston-Salem, North Carolina; and New York, New York). The study was designed to represent a multi-ethnic population, so participants were selected to be African American, Caucasian, Asian (primarily Chinese), or Hispanic. Over the course of approximately ten years of follow-up, subjects participated in measurement of cardiovascular risk factors and questionnaires at five separate visits

("examinations"). Examinations occurred between 2000 and 2002 (examination 1), between 2002 and 2004 (examination 2), between 2003 and 2005 (examination 3), between 2005 and 2007 (examination 4), and finally between 2010 and 2012 (e). An additional follow-up examination (examination 6) occurred later, from 2016-2018.

Mediators: Central Retinal Arteriolar Equivalents and Adhesion Proteins

We chose potential mediators based on their relationship with $PM_{2.5}$ or NO_X in the MESA cohort in previously described analyses (Aims 1 and 2). The chosen mediators were a marker of retinal vessel diameter, central retinal arteriolar equivalents (CRAE), and three circulating markers of adhesion, CCL21, intercellular adhesion molecule 1 (ICAM-1), and tissue inhibitor of metalloproteinase (TIMP-2).

Central Retinal Arteriolar Equivalents (CRAE)

The measurement and significance of this variable has been described previously (Aim 1). Briefly, CRAE is a measure of arteriolar vessel diameters calculated from a photograph of the retina. In MESA, this was calculated by graders using imaging software (IVAN, University of Wisconsin). CRAE was measured at two time points in the MESA cohort. The first "baseline" measurement of CRAE was taken at the second MESA examination which took place from 2002 to 2004 and the single "follow-up" measurement was taken at the fifth MESA examination which took place from 2010 to 2012.

Adhesion Proteins

Adhesion proteins CCL21, ICAM-1, TIMP-2 were measured in serum in blood drawn at the second MESA examination (2002-2004) using enzyme-linked immunosorbent assay (ELISA).

Coronary Artery Calcification

Coronary artery calcification was imaged using computed tomography (CT), calibrated with different density phantoms, and scored using the Agatston method (Carr et al., 2005). CAC was measured at baseline recruitment between 2000 and 2002 (examination 1), then either between 2002 and 2004 (examination 2) or between 2003 and 2005 (examination 3), then again in a small subset of participants between 2005 and 2007 (examination 4), and finally between 2010 and 2012 (examination 5) in slightly more than half the original remaining cohort. Specifically of note is that by design participants received either an examination 2 CT or an examination 3 CT scan but not both.

Exposure

Long-term exposures to $PM_{2.5}$ and NO_X, over two different time periods, were estimated at participant home addresses using a unified likelihood spatiotemporal model designed specifically for MESA to utilize the cohort-specific monitoring that took place in MESA cities (Keller et al., 2015). Briefly, these exposure models utilize smooth spatial and temporal trends, meteorological information, roadway dispersion modeling, and land-use characteristics such as distance from roadway to characterize traffic-related air pollution. For the CRAE mediation analysis, we averaged participant-specific estimates over the period between examinations 2 and 5. For the adhesion protein analyses, we averaged participant-specific estimates over the year

prior to examination 2. These averaging periods were consistent with previous analyses of these variables (Aims 1 and 2).

Mediation Analysis: Overview

We chose to perform mediation analyses for the combinations of air pollutants ($PM_{2.5}$, NO_X) and endothelial-related variables based on results of previous analyses in the MESA cohort. The analysis for CRAE as a mediator is based on longitudinal retinal data (exams 2 and 5) whereas the analysis for the adhesion proteins as mediators are based on cross-sectional protein data (exam 2).

The outcome for both of these mediation analyses is coronary artery calcium which was collected longitudinally. While the approach to estimating mediation is similar for these two sets of analyses, the specific formulations of the statistical model for coronary artery calcium differed substantially and are described in detail below.

Mediation Analysis: Statistical Model

Average causal mediation effects, direct effects, and total effects were estimated with the mediation package in R (Tingley et al., 2013) which uses the potential outcomes framework (Imai et al., 2010). We estimated direct effects which represent the association corresponding to the statistical effect of a unit increment in exposure on the outcome when the mediator is held constant at its observed level. We also estimated the mediation effect which represents the association corresponding to the statistical effect of a unit increment in exposure on the outcome that occurs exclusively through the mediator's associations with exposure and outcome.

Mediation Analysis: Air Pollution, CRAE, and CAC

The mediation analysis for the effect of air pollution on coronary artery calcium via the possible mediator CRAE involves two statistical models: the model for the relationship between air pollution and the mediator (change in CRAE), and the model for air pollution on change over time in the outcome (CAC). CRAE was measured at two time points (once at the 2002-2004 examination and again at the 2010-2012 examination). The relationship between $PM_{2.5}$ and CRAE was modeled longitudinally such that we estimated an association between $PM_{2.5}$ and a change in CRAE between the follow-up and baseline examinations (previously described in aim 1). The relationship between $PM_{2.5}$ and CAC was also modeled longitudinally using the same mixed effects model. The adjustment variables for the $PM_{2.5}$ and CAC change model included those described previously (Kaufman et al., 2016): age at baseline, sex, race/ethnicity, site, type of CT, BMI, exercise, smoking, second-hand smoke, employment, total cholesterol, high density lipoprotein (HDL), triglycerides, lipid lowering medications, socioeconomic status, education, and income. Interaction terms for these variables with time were included to adjust for progression attributable to those variables. We additionally included a term for CRAE, as is necessary for mediation analysis. The adjustment variables for the $PM_{2.5}$ and CRAE model was the same as previously reported in the fully adjusted model (Aim 1).

Several modifications to these models were necessary in order to perform the mediation analysis. In both the model for CRAE and in the model for CAC, the exposure and parameter of interest correspond to the interaction of follow-up time and exposure over the follow-up period. This interaction is the exposure variable for the purpose of this mediation analysis. However, follow-up time between main MESA examination dates and follow-up time between dates of CT

scans were not identical because CT scans were not taken on the same day as basic MESA exams. In order to perform a mediation analysis on our exposure variable of interest (interaction between follow-up time and exposure), it was necessary for this interaction to be identical for both the CRAE analysis (which relies on follow-up time based on the dates of the main MESA examinations) and the CAC analysis (which relies on follow-up time based on the dates CT scans were taken). We chose to use follow-up time corresponding to the dates of MESA examinations for both the CAC and CRAE mixed effects models.

The follow-up structure also differed substantially for CRAE and CAC measurements. CRAE was measured once in 2002-2004 (examination 2) and again in 2010-2012 (examination 5) whereas CAC was measured in most participants at examinations 1 and 5 but only in a subset of participants at examination 2 (participants who did not receive examination 2 CAC measurements received examination 3 CAC measurements). For the purpose of mediation in the context of this mixed effects model, only observations where both mediator and outcome are available are usable. This would exclude any CAC data collected at examinations 1, 3, and 4 from the mediation analysis and would limit the mediation analysis (including the CRAE model) to the half of the cohort present at examination 2 who received CAC measurement at that time point (there were n=5,928 individuals with CRAE measurements at examination 2 versus only n=2,794 individuals with CAC measurements at examination 2).

In order to perform the CRAE/CAC mediation analysis on the approximately the same set of observations used in the original CRAE analysis (aim 1), we selected an alternative *post-hoc* approach to model CAC that allowed inclusion of CAC measurements at examination 3. However, a longitudinal CAC model with outright observations from examinations 2, 3, and 5 would be incompatible with a CRAE model with observations from examinations 2 and 5 only

(observations must be identical when combining longitudinal models for mediation). Therefore, we fit a model for CAC where examination 3 covariate information and CAC measurements were imputed as examination 2 data for individuals missing CAC measurements at examination 2. The resulting CAC model used for the mediation analysis therefore included CAC data taken at examinations 2, 3 and 5 but the examination 3 data was defined as examination 2 data to make this model statistically compatible (for mediation) with the CRAE model which involved only examinations 2 and 5. This analysis allowed more complete use of the collected CAC measurements and avoided excluding from the CRAE mediation analysis nearly half of the cohort (i.e. avoided excluding those who received CAC measurement at examination 3 instead of examination 2).

Mediation Analysis: Air Pollution, adhesion proteins, and CAC

$PM_{2.5}$ in the year prior to a participant's 2002 to 2004 exam was associated with CCL21, TIMP-2 and ICAM-1, and NO_X in the year prior was associated with CCL21 and ICAM-1. The relationship between air pollution and these adhesion markers was modeled cross-sectionally because adhesion markers were only measured at a single time point (at the 2002-2004 examination). Each of these three adhesion proteins was fit as a mediator in separate mediation models for CAC.

Unlike for the CRAE analysis, for the adhesion mediation analysis the association between air pollution and CAC outcome was modeled as cross-sectional or "baseline" repeated measures effect over CAC measured at examinations 1, 2 and 3. This approach was chosen since the adhesion analysis was based on a cross-sectional analysis. Examinations 1, 2 and 3 were

chosen because these CAC measurements were closest in time to the exposure used in the adhesion molecule analyses (year prior to examination 2).

4.3 RESULTS

Cohort Characteristics

Participant characteristics did not differ substantially as a result of exclusion due to missing mediator data or CAC data. Table 4.1 compares subsets of participants at baseline, participants who returned for examination 2, examination 2 participants with CRAE data, and participants with both CRAE data and CAC measurements at examinations 2 or 3. Table 4.2 compares subsets of participants at baseline, participants who returned for examination 2, examination 2 participants who had adhesion markers, examination 2 participants who had adhesion markers and had CAC at examination 1 and either examination 2 or 3. While the overall sample sizes were reduced, the distributions of covariates remained largely unchanged.

Agatston score (CAC) increased by an average of 137 (standard deviation=274) per 7.8 years from CT scans 1 to 5. Average rate of change in CAC differed in the actual subset of data used for the CRAE mediation analysis: over the period from examination 2 to 5 (using imputed values from examination 3), Agatston score increased on average 123 units (standard deviation=248). Pearson correlation between measured CAC measured at different examinations was high: 0.962 for examinations 1 and 3 and 0.985 for examinations 1 and 2.

Average $PM_{2.5}$ exposure over the follow-up period between examinations 2 and 5 in individuals with CRAE and CAC (exams 2 or 3) was 13.3 $\mu g/m^3$ (standard deviation=2.0, 25^{th} percentile=12.5, 75^{th} percentile=14.4). Average $PM_{2.5}$ exposure over the year prior to examination 2 in individuals with adhesion markers and CAC (exams 2 or 3) was 16.2 $\mu g/m^3$ (standard deviation=3.6, 25^{th} percentile=13.9, 75^{th} percentile=19.6). Average NO_X exposure over

the year prior to examination 2 was 48.5 ppb (standard deviation=24.4, 25th percentile=26.2, 75th percentile=68.1).

CRAE and CAC Mediation Analysis: Sensitivity of Coronary Artery Calcium Results to Exclusions

The association between $PM_{2.5}$ exposure and coronary artery calcium varied by subset of participants, by which time-scale was used for follow-up, and by which CAC measurements over various examinations were used. In the original model(Kaufman et al., 2016), each 5 $\mu g/m^3$ increment in $PM_{2.5}$ exposure was associated with an increase of 31.2 Agatston score units over 7.8 years. By contrast, in the model used for the CRAE mediation analysis, each 5 $\mu g/m^3$ increment in $PM_{2.5}$ exposure was associated with an increase of 11.2 Agatston score units for (Table 4.3).

Some of the models for CAC in Table 4.3 used follow-up time based on the date of the primary MESA examination rather than the date of CT scan. For most participants, the difference between these follow-up time over the period between examination 2 and 5 was small. Average difference between CT scan time and MESA exam follow-up time was 0.34 years (25th percentile: 0 years, 75th percentile: 0.43 years) but there were 218 participants whose difference between follow-up times was between 1 year and 1.9 years (the maximum difference observed). The average follow-up time between examinations 2 and 5 was 7.8 years.

CRAE and CAC Mediation Analysis: Sensitivity of CRAE Results to Exclusions

The association between $PM_{2.5}$ exposure in the year prior to examination 2 and CRAE differed from the results reported previously (aim 1) due exclusion of participants missing CAC

data at examinations 2 or 5. We previously reported that in all available participants comparing individuals differing by 5 $\mu g/m^3$ of $PM_{2.5}$ exposure averaged over the follow-up period between their examinations 2 and 5, adjusted change in CRAE differed on average by -1.41 μm per 7.8 years (95% CI: -3.40, 0.58, p=0.17). These results were based on 8,939 observations in 5,521 unique participants.

After restricting to individuals with coronary artery calcium at examination 2, comparing individuals differing by 5 $\mu g/m^3$ of $PM_{2.5}$ exposure averaged over the follow-up period between their examinations 2 and 5, we found that the adjusted change in CRAE differed on average by .07 μm per 7.8 years (95% CI: -3.175, 3.9, p=0.97). These results were based on 3,546 observations in 2,539 unique participants.

After restricting to individuals with coronary artery calcium measurements at examinations 2 or 3 (corresponding to the CAC model where imputed exam 3 values are used for examination 2), comparing individuals differing by 5 $\mu g/m^3$ of $PM_{2.5}$ exposure averaged over the follow-up period between their examinations 2 and 5, we found that the adjusted change in CRAE differed on average by -0.72 μm per 7.8 years (95% CI: -3.14, 1.69, p=0.56). These results were based on 7,152 observations in 4,878 unique participants.

CRAE and CAC Mediation Analysis: Main Mediation Findings

The mediation analysis results for the CRAE/CAC analysis are presented in Table 4.4. These results correspond to change in CAC between examinations 2 and 5 with imputation of examination 3 CAC data. Comparing individuals differing by 5 $\mu g/m^3$ averaged over the follow-up period between examinations 2 and 5, CAC increased on average by 11.1 Agatston score units per 7.8 years (95% CI -38.2, 61.3, p=0.65), adjusting for confounders. The adjusted

mediation effect, corresponding to the association between exposure and outcome describable by the mediator, was 0.2 (95% CI: -0.9, 1.7, p=0.66).

Adhesion Proteins and CAC Mediation Analysis: Main Mediation Analysis

Using a mediation analysis, we found total effects between $PM_{2.5}$ and CAC that were nonsignificant but consistent with the expected direction of effect for all three adhesion marker analyses (Tables 4.5, 5.6, and 4.7). The mediation effects for the TIMP-2 and CCL21 mediation models were significant with relatively large mediation estimates compared to the overall total effects. For mediation of TIMP-2, the adjusted mediation estimate of $PM_{2.5}$ on CAC was 7.5 (95% CI 1.3, 15.7) Agatston score units per 5 $\mu g/m^3$ $PM_{2.5}$ averaged over the year prior to examination 2 compared to a total adjusted estimate of 28.7 (95% CI: -43.4, 102.8) Agatston score units per 5 $\mu g/m^3$ $PM_{2.5}$ averaged over the year prior to examination 2. For mediation of CCL21, the adjusted mediation estimate of $PM_{2.5}$ on CAC was 7.0 (95% CI 0.9, 15.3) Agatston score units per 5 $\mu g/m^3$ $PM_{2.5}$ averaged over the year prior to examination 2 compared to a total adjusted estimate of 27.6 (95% CI: -47.5, 97.9) Agatston score units per 5 $\mu g/m^3$ $PM_{2.5}$ averaged over the year prior to examination 2.

The total effects between NO_X and CAC were negatively associated in the unexpected direction of effect but nonsignificant (Tables 4.8, 4.9, and 4.10). In the CCL21 mediation model for the relationship between NO_X and CAC, the direction of the mediation effect was opposite to the direction of the total and direct effects) (Table 4.10). For mediation of CCL21, the adjusted mediation estimate of NO_X on CAC was 5.5 (95% CI 0.8, 12.5) Agatston score units per 42.1 ppb NO_X averaged over the year prior to examination 2 compared to a adjusted total estimate of -

6.8 (95% CI: -68.4, 52.9) Agatston score units per 42.1 ppb NO_X averaged over the year prior to examination 2.

4.4 DISCUSSION

We observed evidence of statistical mediation by CCL21 and TIMP-2 of the cross-sectional relationship between $PM_{2.5}$ exposure averaged over the year prior to examination 2 and CAC collected at examinations 1, 2 and 3. We also observed evidence of statistical mediation by CCL21 for the cross-sectional relationship between NO_X exposure averaged over the year prior to examination 2 and CAC collected at examinations 1, 2 and 3, although this association was in the opposite direction of the total effect. None of these mediation effects would be significant after accounting for multiple comparisons using a Bonferroni correction, and for none of these mediation analyses was the total effect significant. However, the absence of a significant total effect does not preclude interpretation of mediation effect (Hayes, 2009), especially when we have *a priori* evidence of a relationship between both air pollutants and CAC (Kaufman et al., 2016). It is important to emphasize that the statistical model for CAC used in the mediation analysis for the adhesion proteins was considerably different from the previously published analysis of CAC and air pollution exposure in MESA. Here, we analyzed mediation in terms of a cross-sectional association between the pollutants and CAC at examinations 1, 2, and 3 compared to the original analysis, which modeled progression in CAC over exams 1 through 5. These alterations to the CAC model were chosen to match the exposure used in the adhesion protein analysis. Overall, these results suggest that the proteins CCL21 and TIMP-2 may statistically mediate some of the relationship between CAC and air pollution exposure, but this

result does not necessarily imply causal mediation which relies on strong untestable assumptions discussed below.

We observed no suggestion of a mediation effect via change over time in CRAE on the relationship between $PM_{2.5}$ and change over time in CAC. The model used for CAC in the CRAE mediation analysis, like the originally published CAC model, was a model for progression (change over time) of CAC. However, the CAC analysis used for the CRAE mediation had several modifications from the originally published model: it used information from examinations 2, 3, and 5 only; examination 3 data was imputed to examination 2, and time between MESA examinations was used as the follow-up time variable. Overall, we found no evidence of mediation by CRAE, but the difficult nature of mediation in the context of longitudinal models for both mediator and outcome given temporally misaligned mediator and outcome data, suggest the possibility of bias due to modifications of the CAC model.

The descriptive characteristics in Tables 4.1 and 4.2 suggest that despite the additional exclusions, participant characteristics did not vary substantially from the previously analyzed sub-cohorts for the respective analyses. Despite the absence of changes in demographic information due to subsampling, the main effects of CAC and CRAE were modified due to these exclusions. The estimates of association for air pollution on progression in CAC tended to have similar directions but varying strengths of association. Coronary artery calcium in the original model had an adjusted association of 31.2 Agatston score units over 7.8 years comparing participants differing by 5 $\mu g/m^3$ over the course of follow-up. This is compared to an adjusted association of 11.2 Agatston score units for the model used for the CRAE mediation analysis. Most of this change in the size of the estimates of association was due to differences between the models with all examinations versus the model that involved follow-up over the period from

examination 2 or 3 through 5. Additional sensitivity analyses suggest that the strongest period of follow-up in MESA contributing to the overall estimate of 31.2 Agatston score units was due to the time between examinations 1 and 3 (the estimate for the period between examinations 1 and 3 was 62 with 95% confidence interval of -14.9 to 139.0), but the CAC progression model used here for testing mediation of CRAE did not include data on progression from examinations 1 to 3. It is possible that progression was stronger earlier in follow-up due to age-related phenomena, or this could be the result of random variation due to arbitrary subdivision of the data. It is also worth noting that the estimate differed (from 7.6 to 10.5) when changing the model specification to use the incorrect follow-up time variable which was based on date of examination rather than date of CT scans. This may introduce some bias into the model, but fitting the longitudinal mediation effect using this mixed effects model was not possible without selecting identical time variables for both CRAE and CAC. For most but not all participants, the difference between follow-up times was relatively negligible given the long period of follow-up between examinations 2 and 5.

The CRAE model also had a substantially attenuated estimate after exclusions. The adjusted association previously reported was -1.4 μm over 7.8 years comparing participants differing by 5 $\mu g/m^3$ over the course of follow-up. We initially considered a model using only examination 2 CAC data (without imputation of examination 3 data for individuals missing examination 2 CAC). In this mixed effects model for CRAE, subsetted to individuals nonmissing CAC at examination 2, the association between air pollution and change in CRAE became positive (opposite to the previous result) and effectively null when excluding participants missing coronary artery calcium scores at examination 2. The observed adjusted association was 0.07 μm per 7.8 years (95% CI: -3.175, 3.9, p=0.97) for 5 $\mu g/m^3$, based on only 3,546

observations in 2,539 unique participants. The difference in estimates sizes between these two different samples of the MESA cohort was somewhat surprising because the time of CT scan (exam 2 or 3) was randomized by design of the study. However, the sample size was much smaller compared to the original analysis (which had 8,939 observations in 5,521 unique participants), so an altered result in an arbitrary subsample is not unlikely due to chance alone.

Given the absence of an association between $PM_{2.5}$ and CRAE in the subset of individuals with an examination 2 CT scan, a mediation analysis would not be capable of finding a mediation effect using those results for CRAE in the subset of participants with examination 2 CAC measurements. However, since we had *a priori* evidence suggesting a possible association between air pollution and CRAE based on the results in the full cohort, we performed a mediation analysis in the full cohort by imputation of examination 3 CAC data. After restricting this analysis to participants with CAC at examination 2 or 3, the adjusted association size became -7.3 µm over 7.8 years comparing participants differing by 5 $\mu g/m^3$ over the course of follow-up from examinations 2 to 5. The very high correlation between examination 1 and 2 CAC data (r=0.985) suggests that in MESA Agatston score did not change substantially from one examination to the next and that the use of participants examination 3 CAC scores at examination 2 is reasonable.

This decision to impute examination 3 data was a *post-hoc* modeling decision (based on the absence of results in the subset of individuals with examination 2 CT scans), so even if we did find a positive finding for the CRAE mediation analysis it would be subject to an increased risk of being a spurious finding. Ultimately, we did not find any evidence of CRAE mediating the relationship between $PM_{2.5}$ and CAC using these data.

Unlike for the mediation analysis of CRAE, the adhesion model relied on a previously unreported cross-sectional, repeated measures analysis of the association between air pollution and CAC. Since the adhesion models were cross-sectional with the exposure period defined as the year prior to examination 2, the exposure period for the CAC-adhesion mediation analysis needed to match. Additionally, since the adhesion analysis did not estimate a progression effect (ie there was no interaction by follow-up time), the CAC-adhesion mediation analysis also needed to be a cross-sectional analysis (without interaction by follow-up time). For this CAC-adhesion mediation analysis, we only used CAC measurements from adjacent examinations (1, 2, and 3 but not 4 and 5) for this analysis to select the relevant CAC measurements for this period of exposure. A limitation of this CAC-adhesion mediation analysis was that the examination 1 CAC measure preceded this exposure period of the year prior to examination 2. However, correlation between exposure estimates in the year prior to examinations for adjacent examinations are high (year prior to examinations 1 versus examination 2 Pearson R was 0.98 for NO_X and 0.94 for $PM_{2.5}$) suggesting that examination 2 exposure is an appropriate proxy for exposure over this entire time period. Additionally, since air pollution is an extrinsic exposure, reverse causality is not typically a concern when using an outcome that precedes exposure.

We observed relatively large mediation effects for TIMP-2 and CCL21 in the absence of significant total effects. The total estimate for $PM_{2.5}$ was positively associated with CAC in the expected direction of association whereas NO_X was negatively associated with CAC in the unexpected direction. For both pollutants these total associations had very large confidence intervals. This is in contrast with previous findings in MESA which showed associations of $PM_{2.5}$ and NO_X with CAC progression over all examinations (Kaufman et al., 2016). Associations between historical exposures and recent exposures, latency periods for CAC

progression, and other cohort effects could explain the absence of a precise cross-sectional effect in the presence of a progression effect.

That CCL21 and TIMP-2 had strong mediation associations is relatively unsurprising given that we had already observed strong associations with air pollution exposure and that the relationship of these proteins to cardiovascular disease has been previously demonstrated. TIMP-2 was found to be associated with CAC in a previous analysis of MESA data (Bielinski et al., 2013). CCL21 is elevated in atherosclerotic lesions in humans and in $ApoE^{-/-}$ mice and may be involved in T cell recruitment to lesions (Damås et al., 2007).

However, these mediation results do not provide definitive evidence that these specific proteins are the causal agents between air pollution exposure and cardiovascular disease. The assumptions of mediation analysis limit the interpretation of these results. Specifically there must be no unmeasured confounders between the mediator (adhesion proteins) and the outcome (coronary artery calcification). The assumption of no unmeasured confounding is typical of epidemiologic analyses but is particularly strong in the case of two intrinsic biological variables. A confounder of this relationship could represent any pathway or molecule that causes atherosclerotic lesions and also upregulates these adhesion proteins. Considering that the expression of these proteins could be secondary to the formation of lesions that cause coronary artery calcification, from these results we cannot conclude these proteins are causal agents in air pollution pathobiology.

Specifically, TIMP-2 may not be the causal agent but rather a compensatory response to other processes that lie on the pathway between air pollution exposure and atherosclerotic lesion development. TIMP-2 actually inhibits plaque development in mice (Johnson et al., 2006), so it is more than likely that increased expression of TIMP-2 in humans is a secondary response to

lesion formation. Therefore TIMP-2 is probably not an effective target for intervention but simply reflective of a set of tightly regulated proteins involved in atherosclerotic lesion pathogenesis.

ICAM-1 was not a significant mediator, which means it was not strongly related to coronary artery calcium. This is somewhat surprising given that some observational studies are consistent with ICAM-1 being expressed in plaques or areas prone to plaques. Increased expression of ICAM was found in atherosclerotic lesions compared to non-lesioned areas in arteries (Poston et al., 1992). Increased expression of ICAM was found on endothelial cells adjacent to infiltrated leukocytes (macrophages and T lymphocytes) and the ICAM-1 ligand LFA-1 was found expressed on these leukocytes (van der Wal et al., 1992). However, since ICAM-1 is a surface protein, its circulating levels may not directly reflect pathogenic surface expression. We measured circulating levels of ICAM-1, which limits our ability to detect actual endothelial or atherosclerotic ICAM-1 changes.

Limitations

The previously reported assoication of air pollution with CAC progression was sensitive to subsetting to exam 2 to 5 exposure, especially when restricted to individuals who also had retinal measures or adhesion markers. The modifications necessary to fit the mediation model for CRAE somewhat limit the interpretation of these results and preclude our ability to fully rule out mediation by CRAE. However, a major strength of the CRAE analysis is the longitudinal nature of both mediator and outcome.

The adhesion analysis was limited primarily by its cross-sectional measures of mediator and outcome. The cross-sectional nature of this analysis limits the causal interpretation of these

results since differences in the mediator cannot necessarily be attributed to a period before differences in the outcome. This issue relates directly to the limitation discussed above regarding the possibility of unmeasured confounders for the relationship between adhesion proteins and CAC. Future mediation analyses would benefit from mediator and outcome data collected consistently at the same examination visits in all of the same participants.

Conclusion

Overall, this analysis confirms that the air pollution and CCL21/TIMP-2 associations we observed previously are also related to coronary artery calcification cross-sectionally. This analysis therefore provides increased confidence that these proteins are in fact related to cardiovascular disease in this cohort. However, we cannot rule out the possibility that there are unknown confounders between the mediator and the outcome, so these results do not directly implicate these proteins as pathogenic causal agents. Rather, our results suggest further air pollution research is needed on these proteins, related pathways, and their ability to induce atherosclerotic changes as a result of air pollution. Research in an experimental setting would be well suited to better determine whether the exact pathways involved in upregulation of CCL21 and TIMP-2 jointly involve air pollution and atherosclerosis.

4.5 TABLES

Table 4.1 Participant characteristics and exclusions for the CRAE mediation analysis

	Exam 1	Exam 2 Follow-up	Exam 2 CRAE*	Exam 2 CRAE and 2/3 CAC **
N	6814	6233	5921	5102
CRAE at Exam 2, μm			144 (14)	144 (14)
CRVE at Exam 2, μm			214 (22)	214 (22)
Age at baseline, y	62.2 (10.2)	62 (10.2)	61.5 (10)	61.3 (10)
Age at time of exam, y	62.2 (10.2)	63.6 (10.1)	63.1 (9.9)	62.9 (9.9)
Gender				
Male	3213 (47%)	2969 (48%)	2834 (48%)	2408 (47%)
Female	3601 (53%)	3264 (52%)	3087 (52%)	2694 (53%)
Race/ethnicity				
White	2622 (38%)	2464 (40%)	2352 (40%)	2023 (40%)
Chinese	803 (12%)	728 (12%)	694 (12%)	622 (12%)
Black	1893 (28%)	1691 (27%)	1593 (27%)	1366 (27%)
Hispanic	1496 (22%)	1350 (22%)	1282 (22%)	1091 (21%)
Education				
Less than high school	1225 (18%)	1044 (17%)	974 (16%)	819 (16%)
High school	1236 (18%)	1124 (18%)	1054 (18%)	891 (17%)
Some college/technical	1937 (29%)	1773 (29%)	1689 (29%)	1468 (29%)
College or graduate	2393 (35%)	2274 (37%)	2187 (37%)	1923 (38%)
BMI at baseline, kg/m^2	28.3 (5.5)	28.3 (5.4)	28.3 (5.4)	28.3 (5.4)
BMI at time of exam, kg/m^2	28.338 (5.478)	28.361 (5.498)	28.401 (5.481)	28.366 (5.474)
Smoking at baseline				
Never	3418 (50%)	3142 (51%)	2991 (51%)	2609 (51%)
Former	2487 (37%)	2293 (37%)	2161 (37%)	1859 (36%)
Current	887 (13%)	781 (13%)	753 (13%)	634 (12%)
Smoking at time of exam				
Never	3418 (50%)	2857 (46%)	2721 (46%)	2397 (47%)
Former	2487 (37%)	2637 (43%)	2490 (42%)	2131 (42%)
Current	887 (13%)	695 (11%)	671 (11%)	562 (11%)

Summary values are mean (SD) or count (percentage). *All participants who attended examination 2 and had a measured CRAE value at examination 2. **All participants who attended examination 2, had a measured CRAE value at examination 2, and had measured CAC values at either examination 2 or 3.

Table 4.2 Participant characteristics and exclusions for the adhesion protein mediation analysis

	Exam 1	Exam 2*	Exam 2 Adhesion**	Exam 2 Adhesion & CAC***
N	6814	6233	2569	2218
Age at baseline, y	62.2 (10.2)	62 (10.2)	61.3 (10.1)	61.2 (10)
Age at time of exam, y	62.2 (10.2)	63.6 (10.1)	63 (10)	62.8 (10)
Gender				
Male	3213 (47%)	2969 (48%)	1211 (47%)	1029 (46%)
Female	3601 (53%)	3264 (52%)	1358 (53%)	1189 (54%)
Race/ethnicity				
White	2622 (38%)	2464 (40%)	666 (26%)	566 (26%)
Chinese	803 (12%)	728 (12%)	646 (25%)	580 (26%)
Black	1893 (28%)	1691 (27%)	622 (24%)	527 (24%)
Hispanic	1496 (22%)	1350 (22%)	635 (25%)	545 (25%)
Education				
Less than high school	1225 (18%)	1044 (17%)	526 (21%)	449 (20%)
High school	1236 (18%)	1124 (18%)	466 (18%)	396 (18%)
Some college/technical	1937 (29%)	1773 (29%)	708 (28%)	622 (28%)
College or graduate	2393 (35%)	2274 (37%)	862 (34%)	751 (34%)
BMI at baseline, kg/m^2	28.3 (5.5)	28.3 (5.4)	27.9 (5.5)	27.8 (5.5)
BMI at time of exam, kg/m^2	28.3 (5.5)	28.4 (5.5)	28 (5.5)	27.9 (5.5)
Smoking at baseline				
Never	3418 (50%)	3142 (51%)	1404 (55%)	1231 (56%)
Former	2487 (37%)	2293 (37%)	816 (32%)	697 (31%)
Current	887 (13%)	781 (13%)	342 (13%)	290 (13%)
Smoking at time of exam				
Never	3418 (50%)	2857 (46%)	1282 (50%)	1141 (52%)
Former	2487 (37%)	2637 (43%)	970 (38%)	821 (37%)
Current	887 (13%)	695 (11%)	295 (12%)	251 (11%)
Diabetes at baseline				
Normal	4992 (74%)	4613 (74%)	1856 (72%)	1608 (73%)
Impaired fasting glucose	939 (14%)	855 (14%)	386 (15%)	338 (15%)
Untreated diabetes	179 (03%)	158 (03%)	70 (03%)	64 (03%)
Treated diabetes	680 (10%)	587 (09%)	253 (10%)	207 (09%)
Diabetes at time of exam				
Normal	4992 (74%)	4225 (68%)	1724 (67%)	1489 (67%)
Impaired fasting glucose	939 (14%)	1052 (17%)	442 (17%)	391 (18%)
Untreated diabetes	179 (03%)	166 (03%)	73 (03%)	63 (03%)
Treated diabetes	680 (10%)	745 (12%)	328 (13%)	273 (12%)

Summary values are mean (SD) or count (percentage). *All participants who attended examination 2 **All participants who attended examination 2 and had measured adhesion values ***All participants who attended examination 2 and had measured adhesion values and had measured CAC values at exam 1 and exams 2 or 3.

Table 4.3 Association between average $PM_{2.5}$ exposure and CAC progression; Sensitivity of the association to exclusions used in the CRAE mediation analysis

Exams used	Follow-up Time	Additional Exclusions	N obs	N ppts	Estimate** (95% Confidence Interval)
1,2,3,4,5	CT scan date		16422	6796	31.2 (5.6, 56.8)
2,5	CT scan date		3983	2790	7.6 (-68.1, 83.3)
2,5	Main exam date		3974	2781	10.5 (-69.2, 90.3)
2 (3)*, 5	Main exam date		8024	5334	11.4 (-34.4, 57.1)
2 (3)*, 5	Main exam date	Nonmissing CRAE	7152	4878	11.2 (-37, 59.3)

*Progression from exam 2 to exam 5 using imputed data from exam 3 in individuals missing exam 2 CAC.
** Change in Agatston score units per 7.8 years comparing individuals differing by 5 µg/m3 PM2.5 averaged over the follow-up

Table 4.4 Mediation Results: $PM_{2.5}$, Change in CRAE, and Change in CAC

	Estimate	95% CI Lower	95% CI Upper	p value
Average Causal Mediation Effect	0.2	-0.9	1.7	0.66
Average Direct Effect	10.9	-38.4	60.8	0.67
Total Effect	11.1	-38.2	61.3	0.65

Change in Agatston units between examinations 2 and 5 per 7.8 years comparing individuals differing by 5 $\mu g/m^3$ $PM_{2.5}$ averaged over the follow-up period between examinations 2 and 5.

Table 4.5 Mediation Results: $PM_{2.5}$, TIMP2, and CAC

	Estimate	95% CI Lower	95% CI Upper	p value
Average Causal Mediation Effect	7.5	1.3	15.7	0.01
Average Direct Effect	21.1	-50.2	95.1	0.58
Total Effect	28.7	-43.4	102.8	0.46

Cross-sectional association with Agatston units at examinations 1, 2, and 3 comparing individuals differing by 5 $\mu g/m^3$ $PM_{2.5}$ averaged over the year prior to examination 2.

Table 4.6 Mediation Results: $PM_{2.5}$, ICAM-1, and CAC

	Estimate	95% CI Lower	95% CI Upper	p value
Average Causal Mediation Effect	0.6	-4.7	6.4	0.86
Average Direct Effect	20.5	-53.3	95.8	0.58
Total Effect	21.0	-54.1	95.7	0.57

Cross-sectional association with Agatston units at examinations 1, 2, and 3 comparing individuals differing by 5 $\mu g/m^3$ $PM_{2.5}$ averaged over the year prior to examination 2.

Table 4.7 Mediation Results: $PM_{2.5}$, CCL21, and CAC

	Estimate	95% CI Lower	95% CI Upper	p value
Average Causal Mediation Effect	7.0	0.9	15.3	0.02
Average Direct Effect	20.6	-55.1	91.8	0.58
Total Effect	27.6	-47.5	97.9	0.45

Cross-sectional association with Agatston units at examinations 1, 2, and 3 comparing individuals differing by 5 $\mu g/m^3$ $PM_{2.5}$ averaged over the year prior to examination 2.

Table 4.8 Mediation Results: NO_X, TIMP2, and CAC

	Estimate	95% CI Lower	95% CI Upper	p value
Average Causal Mediation Effect	-0.2	-5.3	4.6	0.91
Average Direct Effect	-11.2	-85.2	57.1	0.74
Total Effect	-11.5	-86.8	56.3	0.74

Cross-sectional association with Agatston units at examinations 1, 2, and 3 comparing individuals differing by 42.1 ppb NO_X averaged over the year prior to examination 2.

Table 4.9 Mediation Results: NO_X, ICAM-1, and CAC

	Estimate	95% CI Lower	95% CI Upper	p value
Average Causal Mediation Effect	0.6	-4.2	5.4	0.78
Average Direct Effect	-13.3	-73.7	48.0	0.66
Total Effect	-12.7	-72.2	49.1	0.69

Cross-sectional association with Agatston units at examinations 1, 2, and 3 comparing individuals differing by 42.1 ppb NO_X averaged over the year prior to examination 2.

Table 4.10 Mediation Results: NO_X, CCL21, and CAC

	Estimate	95% CI Lower	95% CI Upper	p value
Average Causal Mediation Effect	5.5	0.8	12.5	0.01
Average Direct Effect	-12.3	-75.1	48.2	0.74
Total Effect	-6.8	-68.4	52.9	0.85

Cross-sectional association with Agatston units at examinations 1, 2, and 3 comparing individuals differing by 42.1 ppb NO_X averaged over the year prior to examination 2.

Chapter 5. CONCLUSION

Air pollution is a known risk factor for mortality and cardiovascular morbidity (Brook et al., 2010), yet we know relatively little about the processes through which this exposure contributes to cardiovascular disease development. This dissertation focused on a set of specific biologic measures, retinal microvasculature and circulating adhesion-related proteins, in order to determine whether air pollution could alter the pathways that these measures represent.

Overall our results suggest a positive association between air pollution exposure and vascular and adhesion processes. In aim 1 we found that change in CRAE had a suggestive but non-significant association with $PM_{2.5}$ exposure as well as suggestive associations with acute exposures. The association between long-term $PM_{2.5}$ exposure and change in CRAE was relatively large; approximately half of the average change over time in CRAE over the follow-up period. In aim 2 we found that CCL21, ICAM-1, and TIMP-2 were strongly associated with long-term $PM_{2.5}$ exposure cross-sectionally and that CCL21 and ICAM-1 were associated with NO_X. We found relatively weak or null associations between acute $PM_{2.5}$ exposure and the adhesion proteins. Finally in aim 3 we found that the associations of TIMP-2 and CCL21 with air pollution could in part possibly mediate a positive relationship between air pollution exposure and coronary artery calcium, although this overall relationship was not significant and the strong possibility of unknown confounders between adhesion proteins and coronary artery calcium limits our ability to conclude whether these proteins are causal agents.

Our results from aim 1 are consistent with a small number of existing studies on the relationship between air pollution exposure and measures of retinal vascular caliber. A previous analysis of the examination 2 data in MESA found an association between CRAE and both acute and long-term $PM_{2.5}$. Outside of MESA, one group found an association between day prior black

carbon and PM_{10} and decreases in CRAE as well as increases in CRVE using a repeated measures design (Louwies et al., 2013). This group replicated these results in adults for PM_{10} in another study (Louwies et al., 2016), and also have shown a relationship between $PM_{2.5}$ and CRAE in children (Provost et al., 2015, 2017). Only one of these analyses considered long-term exposure and found a negative but nonsignificant relationship between long-term CRAE and $PM_{2.5}$ (Provost et al., 2017). While some of these studies have involved repeated measures, to date there have been no other analyses of the relationship between air pollution exposure and change over time in retinal measures of vascular caliber. The use of longitudinal change in our analyses is a major strength as it provides increased plausibility that the observed relationship is not due to bias or confounding. Nevertheless, further research is needed, especially on long-term exposure and whether vascular changes represented by the association between air pollution and CRAE mediate the relationship between air pollution and cardiovascular disease.

While we were not able to find evidence of mediation by CRAE in our analysis, there is substantial and well-replicated evidence showing that CRAE and related arteriolar diameter measures are associated with risk of cardiovascular-related mortality (Seidelmann et al., 2016; Witt et al., 2006; Wong et al., 2003). This result often persists even after adjusting for traditional risk factors including blood pressure. These findings strongly suggest that differences in arteriolar diameters do in fact reflect vascular changes that are characteristic of cardiovascular disease. It is possible that changes in CRAE are not associated with calcification but other vascular alterations that increase risk of cardiovascular events. For example, CRAE might reflect reduced endothelial function and reactive hyperemia, which could exacerbate occlusions when they occur. Or it is possible that even if air pollution is causally associated with CRAE, the alteration of CRAE due to air pollution is not the mechanism through which air pollution

increases risk of cardiovascular events. Further research is needed on an expanded set of cardiovascular related outcomes such as survival to determine whether observed associations between air pollution and CRAE are pathologically meaningful.

Whether CRAE reflects cardiovascular changes is an area of potential research. Arteriolar diameter may be associated with measures of myocardial blood flow (Wang et al., 2008). However, this result was only observed in individuals without any coronary artery calcium. This does not necessarily imply CRAE-related perfusion deficiency and atherosclerotic CAC are mutually exclusive effects. Coronary artery calcium induced stenosis may simply alter blood-flow so substantially that microvascular effects on perfusion are harder to detect in individuals with advanced CAC. Nevertheless, the extent to which CRAE acts as an adequate proxy for coronary microvasculature is largely unanswered.

More broadly, there is insufficient research on exactly what the association between CRAE and CVD events means with regard to cardiovascular disease biology. Does CRAE reflect an atherosclerotic phenotype? Does CRAE simply precede later changes in blood pressure, which in turn increase risk of CVD events? Or does CRAE reflect a unique vascular phenotype—independent of blood pressure—that involves endothelial dysfunction, dysregulation of coronary blood flow, or thrombosis which might exacerbate the effects of atherosclerotic stenosis? These questions might be addressed using serial observational data, but no epidemiologic study to date has addressed them directly. Future research in this area might involve analyses on the association between CRAE and CVD events to determine whether this effect is mediated by changes in blood pressure, coronary artery calcium, measures of endothelial function, and measures of inflammation.

Additionally, there is a very unique and abnormal characteristic of retinal arterioles: they lack sympathetic innervation. This difference from typical systemic and myocardial vessels makes it particularly difficult to theorize on how observational changes in CRAE related to other vascular beds such as the myocardium. This may be a disadvantage for use in observation research, but it may actually be a strength for human controlled exposure studies. Acute changes in retinal vessels presumably reflects local factors and circulating systemic factors but not direct sympathetic innervation. In fact, we have seen paradoxical widening of retinal arterioles in conjunction with increased blood pressure as a result of controlled diesel exposure. One possible avenue for further research would be a simultaneous measurement of CRAE and another measure of endothelial function during controlled exposure—a comparison of these results may allow us to tease out sympathetic response from global compensatory vasodilation.

There are a number of acute controlled exposure studies of air pollution exposure and other measures of endothelial function. Acute exposure to diesel exhaust or concentrated ambient pollutants causes rapid transient increases in blood pressure (Cosselman et al., 2012; Morishita et al., 2015; Urch et al., 2005). Recent controlled exposure research suggest that this blood pressure response is attenuated by an alpha 1 adrenergic blocker as well as by a genetic variant that reduces effectiveness of nociceptive receptor TRPV1. As for endothelial-related effects of air pollution, Mills et al showed that diesel exhaust attenuated response to vasodilators that act through NO (Mills et al., 2005). These results suggest that ROS from air pollution may alter vessel tone through by inhibiting the ability of NO to act on vascular smooth muscle cells. In another study, diesel exhaust caused reduced brachial artery diameter but caused no significant change post versus pre flow-mediated dilation (Peretz et al., 2008), suggesting that air pollution does not directly inhibit endothelial synthesis of NO on this time-scale. These results do not rule

out the possibility that air pollution decreases NO bioavailability acutely during exposure, and does not rule out the possibility that air pollution alters endothelial function over longer periods of exposure. In fact, during two week-long periods of a placebo controlled cross-over study, air filtration was associated with increased reactive hyperemia measured via peripheral artery tonometry (Allen et al., 2011). The same endpoint was associated with filtration during another cross-over study occurring over two 48 hour periods (Bräuner et al., 2008). Reactive hyperemia via peripheral artery tonometry was not associated exposure status in a 3 hour controlled exposure study, but was associated with day prior ambient exposure (III et al., 2011). Overall, human experimental research is consistent with an association between air pollution exposure and an acute blood pressure response, possibly mediated by sympathetic activation, as well as a subacute endothelial response. Whether this subacute endothelial response is mediated by acute sympathetic response could be addressed analyzing genetic variants in combination with placebo cross-over studies of filtration and endothelial function. Notably filtration trials are feasibly able to be of longer duration than controlled exposure studies and results from this design has demonstrated air pollution induced endothelial changes that have not been observed during acute controlled exposure.

More broadly, our study is consistent with prior research on the relationship between air pollution and vascular and endothelial changes. The relationship between air pollution exposure and blood pressure has been well documented (Brook and Rajagopalan, 2009). In MESA, long-term exposure to $PM_{2.5}$ was associated with impaired? flow-mediated dilation (Krishnan et al., 2012b), suggesting that chronic exposure, unlike acute exposure, leads to endothelial dysfunction. Yet relatively little research has been performed on air pollution and longitudinal

measures of endothelial function. Further research on longitudinal changes in other endothelial measures may be useful in interpreting the results we observed with respect to change in CRAE.

Epidemiologic studies also show mixed associations with adhesion molecules. Circulating ICAM-1 and VCAM-1 were associated with a seven day moving average of $PM_{2.5}$ exposure in the Normative Aging Study (Wilker et al., 2011). However, several other studies have shown no association between $PM_{2.5}$ and ICAM-1 (Madrigano et al., 2010; Niu et al., 2013). More broadly, there are several epidemiologic analyses suggesting a relationship between air pollution exposure and less specific inflammatory markers such as CRP(Allen et al., 2011; Pope et al., 2004), IL6 (Rückerl et al., 2007).

There is limited and conflicting evidence in controlled exposure studies regarding the effect of air pollution on adhesion makers. One study showed small and nonsignificant increases in circulating ICAM-1 and VCAM-1 as a result of diesel exhaust exposure compared to filtered air (Krishnan et al., 2013). Conversely, in another study ICAM-1 expression and CD18 expression on monocytes was decreased in response to untrafine particle inhalation (Frampton et al., 2006). A controlled diesel exposure trial showed no association between exposure and P-selectin or IL-6 (Nightingale et al., 2000). On the other hand, considerable in-vitro evidence also links $PM_{2.5}$ exposure to relatively large changes ICAM expression (Ishii et al., 2005; Montiel-Dávalos et al., 2007). Overall there is limited evidence of a circulating adhesion or inflammatory response to acute controlled exposure, but somewhat stronger evidence for pulmonary inflammation (Behndig et al., 2006; Stenfors et al., 2004). Our results indicated much stronger and consistent associations across a wide variety of adhesion proteins for long-term $PM_{2.5}$ exposure compared to acute $PM_{2.5}$ exposure. The controlled exposure literature does not strongly support our findings with regard to ICAM-1, and to our knowledge there are no

controlled exposure studies assessing acute changes in CCL21 or TIMP-2. Further research is needed to determine whether these findings can be replicated in an acute controlled exposure setting.

Whether we can expect to find acute associations between air pollution exposure and adhesion protein changes is unknown. If air pollution induces a pulmonary inflammatory response, it would be theoretically possible for adhesion proteins to be upregulated 24 hours after exposure but inflammatory effects may also act on slower timeframes. Alternatively, adhesion changes may result from much slower pathways—such as vascular remodeling due to chronic exposure to increased blood pressure. Further research is needed to verify and explain whether air pollution actually causes increases in these specific proteins and what the time-frame of exposure and latency periods are. Analysis of blood in controlled exposure studies can at least help identify whether these responses are acute or delayed.

The relationship between changes in CRAE and adhesion molecules is not known, but vessel tone and adhesion expression could be mediated by related pathways. Air pollution may induce increased reactive oxygen species (Brook et al., 2010) and particulate matter itself is oxidative (Janssen et al., 2014). Reactive oxygen species interferes with endothelial NO production and therefore endothelial-dependent vasodilation (Touyz and Schiffrin, 2004) and may also be involved in expression of adhesion molecules (Chiarugi et al., 2003; Chiu et al., 1997). Alternatively, these two pathways may share transcription factors that are activated by air pollution-induced pulmonary inflammation. Nuclear factor kappa-B is involved in regulating both endothelial nitric oxide synthase (Grumbach et al., 2005) as well as ICAM-1 (Rui et al., 2016) and TIMP-2 (Valiño-Rivas et al., 2016). Pathway analyses such as transcriptional

regulatory network analyses may provide additional insight into shared upstream mechanisms of apparently disparate air pollution effects.

Our analysis was limited by a number of characteristics. A disadvantage to any modeled air pollution exposure is the possibility for measurement error. Classical non-differential measurement error may bias the results towards the null. However, in the context of air pollution exposure estimation another type of error known as Berkson-like error can arise due to area-level averaging or smoothing of true exposures and may result in bias away from the null (Szpiro et al., 2011). Further research or sensitivity analyses may be warranted to verify these results are robust to the effects of measurement error.

A related but different limitation is the non-separability of the effects of acute and long-term exposure to air pollution given the exposure approach used in our study. Since acute exposures use a single central-site monitor, exposure contrast from this analysis comes from daily variation in exposure and the fact that participants had examinations on different days. Long-term exposure comes largely from spatial contrasts in participant locations. Due to the different assumptions involved in these exposure estimates and their reliance on different sources of exposure contrast, concurrent adjustment for these effects would not necessarily separate out acute from chronic effects.

Another potential limitation to our analysis is the generalizability of the retinal measures and adhesion proteins to myocardial vascular beds. Retinal vessels lack sympathetic innervation so changes in these vessels behave differently from myocardial vessels. Additionally, circulating measures of adhesion molecules do not express surface or endothelial tissue expression. Therefore, circulating levels reflect non-specific changes that could be coming a variety of

tissues. Additional research in cell lines or mouse models could be used to determine whether air pollution induces expression changes at tissues of interest such as the vascular endothelium.

A major strength of this dissertation was the use of data collected in the Multi-Ethnic Study of Atherosclerosis. MESA's design allows for longitudinal collection of clinical measures such as coronary artery calcium as well as subclinical measures of CRAE. To our knowledge, this is the first analysis of air pollution exposure and long-term change in this measure. This was also the first analysis of TIMP-2 and CCL21 with respect to air pollution exposure in humans. Finally, the MESA spatiotemporal air pollution model allows for relatively accurate air pollution exposure estimates, which reduces the likelihood of measurement error and potentially increases the probability of detecting associations.

In summary, our results support the hypothesis that air pollution exposure may be implicated in vascular and adhesion changes. Studies such as this that provides biologically plausible pathway effects of air pollution contribute to the body of research necessary for setting and maintaining Environmental Protection Agency National Ambient Air Quality Standards. While this research supports evidence of biological plausibility, further research is needed to validate these results and determine whether vascular and adhesion changes can explain the relationship between air pollution and cardiovascular mortality.

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