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ABOUT HEI

The Health Effects Institute is a nonprofit corporation chartered in 1980 as an independent research organization to provide high-quality, impartial, and relevant science on the effects of air pollution on health. To accomplish its mission, the institute

- Identifies the highest-priority areas for health effects research;
- Competitively funds and oversees research projects;
- Provides intensive independent review of HEI-supported studies and related research;
- Integrates HEI's research results with those of other institutions into broader evaluations; and
- Communicates the results of HEI's research and analyses to public and private decision makers.

HEI typically receives balanced funding from the U.S. Environmental Protection Agency and the worldwide motor vehicle industry. Frequently, other public and private organizations in the United States and around the world also support major projects or research programs. HEI has funded more than 340 research projects in North America, Europe, Asia, and Latin America, the results of which have informed decisions regarding carbon monoxide, air toxics, nitrogen oxides, diesel exhaust, ozone, particulate matter, and other pollutants. These results have appeared in more than 260 comprehensive reports published by HEI, as well as in more than 2,500 articles in the peer-reviewed literature.

HEI's independent Board of Directors consists of leaders in science and policy who are committed to fostering the public–private partnership that is central to the organization. The Research Committee solicits input from HEI sponsors and other stakeholders and works with scientific staff to develop a Five-Year Strategic Plan, select research projects for funding, and oversee their conduct. The Review Committee, which has no role in selecting or overseeing studies, works with staff to evaluate and interpret the results of funded studies and related research. For the MOSES initiative, a special MOSES Review Panel — comprising Review Committee members and outside experts — fulfilled this role.

All project results and accompanying comments by the Review Committee (or, in this case, the MOSES Review Panel) are widely disseminated through HEI's website (*www.healtheffects.org*), printed reports, newsletters and other publications, annual conferences, and presentations to legislative bodies and public agencies.

ABOUT THIS REPORT

Research Report 192, Part 2, *Effects of Personal and Ambient Concentrations of Ozone and Other Pollutants on Cardiovascular and Pulmonary Function*, presents a research project funded by the Health Effects Institute and conducted by Drs. David Q. Rich and Mark W. Frampton of the University of Rochester Medical Center, Rochester, New York, and their colleagues. The report contains three main sections.

The HEI Statement, prepared by staff at HEI, is a brief, nontechnical summary of the study and its findings; it also briefly describes the MOSES Review Panel's comments on the study.

The Investigators' Report, prepared by Rich and Frampton and colleagues, describes the scientific background, aims, methods, results, and conclusions of the study.

The Commentary, prepared by members of the MOSES Review Panel with the assistance of HEI staff, places the study in a broader scientific context, points out its strengths and limitations, and discusses remaining uncertainties and implications of the study's findings for public health and future research.

This report has gone through HEI's rigorous review process. The investigators submitted a draft final report, which was evaluated by the HEI MOSES Review Panel — an independent panel of distinguished scientists, which included some members of the HEI Review Committee, all of whom had no involvement in selecting or overseeing this study. Comments from the Panel were sent to the investigators, who revised their report as they considered appropriate. The Commentary by the MOSES Review Panel reflects the information provided in the final version of the report.

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[1] The MOSES Oversight Committee provided input during the study design phase. The HEI Research Committee provided oversight while the study was ongoing.

[2] The MOSES Data Monitoring Board monitored the development and implementation of the data analysis plan, overall quality and management of the data, and subject safety through frequent reviews of adverse event reports. The Board received regular reports from the data managers and statisticians at the New England Research Institute.

[3] The MOSES Review Panel performed independent peer review of the Investigators' Report and prepared the HEI Commentary accompanying the Report.

HEI STATEMENT

Synopsis of Research Report 192, Part 2

Prior Air Pollutant Exposures and Cardiorespiratory Effects of Ozone

INTRODUCTION

Ozone exposure has been associated with adverse health effects in children and adults at current ambient concentrations. Its effects on the respiratory system are well established and include worsening of asthma symptoms (acute effects), increases in deaths and hospital admissions for respiratory illnesses such as chronic obstructive pulmonary disease and asthma (acute and chronic effects), reduced lung growth, and higher risk of developing asthma (chronic effects). Some recent studies have reported that short-term exposure to ozone is associated with adverse cardiovascular outcomes, including an increased risk of cardiovascular mortality.

Ozone is an oxidant gas that easily reacts with other molecules. After inhalation, ozone reacts with constituents of the lung lining fluid to generate reactive oxygen species that can cause local oxidative stress in the lung and lead to lung irritation. With repeated exposure, oxidative stress may lead to lung injury and chronic lung disease. Ozone may have effects on the cardiovascular and other organ systems through systemic inflammation, oxidative stress, or changes in activity of the autonomic nervous system, which could lead to changes in heart rhythm, endothelial dysfunction, constriction of arteries, and blood clotting.

APPROACH

In 2010, HEI funded the Multicenter Ozone Study in oldEr Subjects (MOSES), conducted at three clinical centers in California, North Carolina, and New York. From 2012 through 2015, the investigators used a common protocol to expose 87 healthy

What This Study Adds

- The previously published MOSES study (Part 1) found that controlled ozone exposure at concentrations similar to the current U.S. air quality standard was not associated with changes in cardiovascular endpoints in 87 healthy, older adults, but there were moderate adverse effects on lung function and two markers of lung injury and inflammation.
- The MOSES, Part 2 study in the current report presents additional analyses to evaluate whether the MOSES 1 results were influenced by exposure to ambient air pollutants up to 4 days prior to the controlled ozone exposures. It also evaluated whether the prior exposures were associated with changes in baseline levels of biomarkers.
- MOSES 1 provided confirmation of ozone effects on the lung at low concentrations (70 and 120 ppb). MOSES 2 showed that those results were not affected by prior exposure to ambient pollutants. However, ambient concentrations of ozone and other pollutants were associated with differences in baseline levels of several biomarkers.
- The results of the MOSES studies add to the body of evidence of changes in health outcomes associated with air pollutant exposures at the current — relatively low — ambient concentrations in the United States.

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Drs. David Q. Rich and Mark W. Frampton, Pulmonary & Critical Care, University of Rochester Medical Center, Rochester, NY, and colleagues. Research Report 192, Part 2 contains both the detailed Investigators' Report and a Commentary on the study prepared by the MOSES Review Panel.

volunteers (ages 55–70 years) to 0, 70, and 120 ppb ozone. Exposures lasted 3 hours, during which the participants exercised on a stationary bicycle, alternating 15 minutes of exercise with 15 minutes of rest. Participants stayed at a hotel the night before testing to minimize variability in exposure to ambient air pollutants and were evaluated the day before, during, and up to 22 hours after exposure.

In the previously published MOSES report (Research Report 192, Part 1), the investigators measured a large suite of endpoints, including changes in autonomic nervous system function, heart rhythm, blood pressure, and pulmonary function, as well as markers of endothelial function, thrombosis, lung injury, and both systemic and lung inflammation. They specified in advance a key group of cardiovascular endpoints as primary; all other endpoints were secondary. Results were analyzed by mixed-effects linear models, adjusting for the three centers and multiple time points, and presented as the difference between pre-exposure and post-exposure values. The statistical significance threshold was set at $P < 0.01$ in light of multiple comparisons.

Because the controlled exposure concentrations were close to ambient ozone concentrations experienced every day, there was considerable interest in evaluating whether ambient exposures to ozone and other pollutants during the days leading up to the clinical visits may have influenced the outcome of the experiments. Therefore, the investigators measured each participant's exposure to ozone and nitrogen dioxide using a personal sampler for 72 hours before the pre-exposure visit. They also collected air quality data for ozone, fine particulate matter, nitrogen dioxide, sulfur dioxide, and carbon monoxide from central monitors closest to each clinical center.

MOSES, Part 2 describes analyses conducted by the team at the University of Rochester, who generally used the same statistical approach as in MOSES 1. They ran the statistical models with inclusion of personal exposure measurements for ozone or nitrogen dioxide, or ambient concentrations of each pollutant at various time lags (from 0 to 96 hours prior) for a total of 37 statistical models per biomarker. They also conducted several sets of sensitivity analyses.

The investigators pursued four specific aims: to investigate (1) whether any changes in biomarkers before and after the controlled ozone exposures were *confounded* by prior exposures to ambient air pollutants; (2) whether there was *effect modification*, that is, whether controlled ozone effects could only be seen when prior ambient exposures were low or, alternatively, when they were high; (3) whether prior pollutant exposures were associated with differences in *baseline values* of the biological markers measured before the start of the controlled ozone exposures; and (4) whether prior pollutant exposures were associated with *changes in biomarkers* before and after controlled ozone exposure.

KEY RESULTS

As reported in MOSES 1, there was no evidence that a 3-hour exposure to 70 or 120 ppb ozone with moderate exercise affected cardiovascular endpoints in these healthy older adults. However, short-term exposures at these low ozone concentrations did produce pulmonary effects. In MOSES 2, the investigators found no evidence of confounding by prior exposures to ozone or other air pollutants. They also found no evidence of effect modification when the results were analyzed by tertile of ambient pollutant concentrations, except for changes in lung function. Specifically, changes in forced expiratory volume in one second and in forced vital capacity were observed when carbon monoxide and ambient or personal nitrogen dioxide concentrations were in the medium and highest tertiles (Statement Figure). Although there was some variation in the level of statistical significance across these comparisons, the pattern of changes appeared to be coherent. The investigators hypothesized that prior exposures to these pollutants may have sensitized or primed the airways to respond to the controlled ozone exposures.

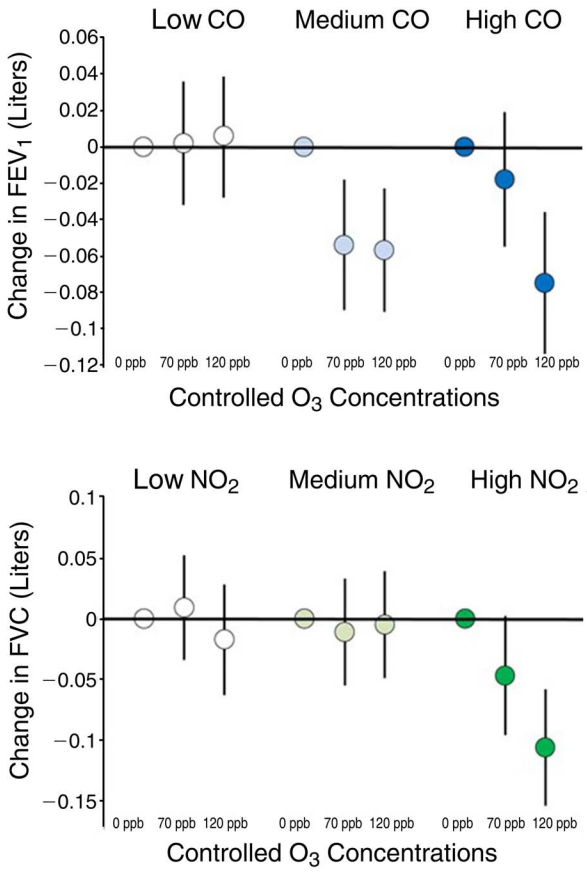
The investigators reported possible associations between ambient ozone exposure and baseline heart rate variability in the frequency domain. There were also possible associations between ambient concentrations of fine particulate matter, carbon monoxide, and nitrogen dioxide and baseline C-reactive protein levels or lung function measures. On the other hand, possible associations of ambient ozone with high-frequency-power heart rate variability were independent of ambient concentrations of fine particulate matter, carbon monoxide, and nitrogen dioxide.

REVIEW PANEL'S EVALUATION

In its independent review of the study, the MOSES Review Panel, specially convened by the HEI Review Committee, commended the investigators for a well-designed and well-executed follow-on study to MOSES 1. In addition to evaluating possible confounding of MOSES 1 results, they evaluated various other research questions to understand how daily ambient pollutant exposures may have affected baseline levels of biomarkers and whether the pollutants interacted with each other. The Panel commended the investigators for conducting a large number of informative statistical analyses in MOSES 2 and agreed with the report's main conclusion that the MOSES 1 results were not confounded by the participants' prior exposures to air pollutants.

The Panel made additional observations on the results and their interpretation. By using an interaction term in MOSES 2, the analysis no longer compared outcomes within each person, because each visit to the clinic may have been preceded by a different ambient pollutant concentration. Thus, the strength of the original crossover design in MOSES 1 no longer applied. The Panel also expressed some concern about multiple testing (37 statistical analyses per biomarker) potentially yielding false positive associations.

The Panel thought the analyses of prior ambient pollutant exposures on baseline levels of the cardiovascular biomarkers (Aim 3) were interesting and the results were consistent with current knowledge. However, the Panel found the analyses for Aim 4 difficult to interpret. The fact that the direction of effects for frequency-domain heart rate variability was inconsistent decreased confidence in the interpretation that prior exposure to ambient ozone may have affected heart rate variability.



Statement Figure. Influence of ambient concentrations during preceding days on changes in lung function after controlled ozone exposure. Ambient pollutant concentrations up to 72 hours prior to ozone exposure were divided into tertiles. Top panel: forced expiratory volume in one second and ambient carbon monoxide. Bottom panel: forced vital capacity and ambient nitrogen dioxide.

CONCLUSIONS

The Multicenter Ozone Study in oldEr Subjects was a large, well-conducted study in 87 healthy adults (55–70 years old). MOSES 1 showed the following important results: (1) there was no convincing evidence that a 3-hour exposure to near ambient concentrations of 70 or 120 ppb ozone with moderate exercise resulted in statistically significant changes in cardiovascular endpoints in these healthy older adults; (2) short-term exposures at these relatively low ozone concentrations did lead to pulmonary effects, consistent with previous studies, which were conducted primarily in younger adults; and (3) no susceptible subgroups could be identified in which ozone elicited cardiovascular effects that were not evident in the group as a whole. MOSES 2 showed that these results were not affected by the participants' immediate prior exposures to ambient air pollutants, providing confidence in the results. The MOSES Review Panel agreed with the main findings of the study and that the results support the conclusion that adverse lung effects can be observed at ozone concentrations resembling the current 8-hour U.S. National Ambient Air Quality Standard (NAAQS) of 70 ppb.

It remains possible that ozone may lead to cardiovascular effects in more susceptible individuals, following longer exposures, or in the presence of common ambient air pollutants. MOSES 2 presented evidence that ambient air pollution exposure may be associated with changes in baseline levels of some cardiovascular and pulmonary biomarkers measured before the clinical visits. These results add to the body of evidence of changes in health outcomes associated with air pollutant exposures at the current — relatively low — ambient concentrations in the United States.

INVESTIGATORS' REPORT

Multicenter Ozone Study in oldEr Subjects (MOSES): Part 2. Effects of Personal and Ambient Concentrations of Ozone and Other Pollutants on Cardiovascular and Pulmonary Function

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ABSTRACT

Introduction The Multicenter Ozone Study of oldEr Subjects (MOSES*) was a multi-center study evaluating whether short-term controlled exposure of older, healthy individuals to low levels of ozone (O_3) induced acute changes in cardiovascular biomarkers. In MOSES Part 1 (MOSES 1), controlled O_3 exposure caused concentration-related reductions in lung function with evidence of airway inflammation and injury, but without convincing evidence of effects on cardiovascular function (Balmes et al 2019). However, subjects' prior exposures to indoor and outdoor air pollution in the few hours and days before each MOSES controlled O_3 exposure may have independently affected the study biomarkers and/or modified biomarker responses to the MOSES controlled O_3 exposures.

Methods MOSES 1 was conducted at three clinical centers (University of California San Francisco, University of North Carolina, and University of Rochester Medical Center) and included healthy volunteers 55 to 70 years of age. Consented participants who successfully completed the screening and training sessions were enrolled in the study. All three clinical centers adhered to common standard operating procedures and used common tracking and data forms. Each subject was scheduled to participate in a total of 11 visits: screening visit, training visit, and three sets of exposure visits consisting of the pre-exposure day, the exposure day, and the post-exposure day. After completing the pre-exposure day, subjects spent the night in a nearby hotel. On exposure days, the subjects were exposed for 3 hours in random order to 0 ppb O_3 (clean air), 70 ppb O_3, and 120 ppm O_3. During the exposure period the subjects alternated between 15 minutes of moderate exercise and 15 minutes of rest. A suite of cardiovascular and pulmonary endpoints was measured on the day before, the day of, and up to 22 hours after each exposure.

In MOSES Part 2 (MOSES 2), we used a longitudinal panel study design, cardiopulmonary biomarker data from MOSES 1, passive cumulative personal exposure samples (PES) of O_3 and nitrogen dioxide (NO_2) in the 72 hours before the pre-exposure visit, and hourly ambient air pollution and weather measurements in the 96 hours before the pre-exposure visit. We used mixed-effects linear regression and evaluated whether PES O_3 and NO_2 and these ambient pollutant concentrations in the 96 hours before the pre-exposure visit confounded the MOSES 1 controlled O_3 exposure effects on the pre- to post-exposure

This Investigators' Report is one part of Health Effects Institute Research Report 192 Part 2, which also includes a Commentary by the MOSES Review Panel and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. David Q. Rich, Department of Public Health Sciences, 265 Crittenden Boulevard, CU 420644, Rochester, New York; e-mail: *David_Rich@URMC.Rochester.edu*.

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* A list of abbreviations and other terms appears at the end of this volume.

biomarker changes (Aim 1), whether they modified these pre- to post-exposure biomarker responses to the controlled O_3 exposures (Aim 2), whether they were associated with changes in biomarkers measured at the pre-exposure visit or morning of the exposure session (Aim 3), and whether they were associated with differences in the pre- to post-exposure biomarker changes independently of the controlled O_3 exposures (Aim 4).

Results Ambient pollutant concentrations at each site were low and were regularly below the National Ambient Air Quality Standard levels. In Aim 1, the controlled O_3 exposure effects on the pre- to post-exposure biomarker differences were little changed when PES or ambient pollutant concentrations in the previous 96 hours were included in the model, suggesting these were not confounders of the controlled O_3 exposure/biomarker difference associations. In Aim 2, effects of MOSES controlled O_3 exposures on forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) were modified by ambient NO_2 and carbon monoxide (CO), and PES NO_2, with reductions in FEV_1 and FVC observed only when these concentrations were "Medium" or "High" in the 72 hours before the pre-exposure visit. There was no such effect modification of the effect of controlled O_3 exposure on any other cardiopulmonary biomarker.

As hypothesized for Aim 3, increased ambient O_3 concentrations were associated with decreased pre-exposure heart rate variability (HRV). For example, high frequency (HF) HRV decreased in association with increased ambient O_3 concentrations in the 96 hours before the pre-exposure visit (−0.460 ln[ms^2]; 95% CI, −0.743 to −0.177 for each 10.35-ppb increase in O_3; $P = 0.002$). However, in Aim 4 these increases in ambient O_3 were also associated with increases in HF and low frequency (LF) HRV from pre- to post-exposure, likely reflecting a "recovery" of HRV during the MOSES O_3 exposure sessions. Similar patterns across Aims 3 and 4 were observed for LF (the other primary HRV marker), and standard deviation of normal-to-normal sinus beat intervals (SDNN) and root mean square of successive differences in normal-to-normal sinus beat intervals (RMSSD) (secondary HRV markers).

Similar Aim 3 and Aim 4 patterns were observed for FEV_1 and FVC in association with increases in ambient PM with an aerodynamic diameter ≤ 2.5 μm ($PM_{2.5}$), CO, and NO_2 in the 96 hours before the pre-exposure visit. For Aim 3, small decreases in pre-exposure FEV_1 were significantly associated with interquartile range (IQR) increases in $PM_{2.5}$ concentrations in the 1 hour before the pre-exposure visit (−0.022 L; 95% CI, −0.037 to −0.006; $P = 0.007$), CO in the 3 hours before the pre-exposure visit (−0.046 L; 95% CI, −0.076 to −0.016; $P = 0.003$), and NO_2 in the 72 hours before the pre-exposure visit (−0.030 L; 95% CI, −0.052 to −0.008; $P = 0.007$). However, FEV_1 was not associated with ambient O_3 or sulfur dioxide (SO_2), or PES O_3 or NO_2 (Aim 3). For Aim 4, increased FEV_1 across the exposure session (post-exposure minus pre-exposure) was marginally significantly associated with each 4.1-ppb increase in PES O_3 concentration (0.010 L; 95% CI, 0.004 to 0.026; $P = 0.010$), as well as ambient $PM_{2.5}$ and CO at all lag times. FVC showed similar associations, with patterns of decreased pre-exposure FVC associated with increased $PM_{2.5}$, CO, and NO_2 at most lag times, and increased FVC across the exposure session also associated with increased concentrations of the same pollutants, reflecting a similar recovery. However, increased pollutant concentrations were not associated with adverse changes in pre-exposure levels or pre- to post-exposure changes in biomarkers of cardiac repolarization, ST segment, vascular function, nitrotyrosine as a measure of oxidative stress, prothrombotic state, systemic inflammation, lung injury, or sputum polymorphonuclear leukocyte (PMN) percentage as a measure of airway inflammation.

Conclusions Our previous MOSES 1 findings of controlled O_3 exposure effects on pulmonary function, but not on any cardiovascular biomarker, were not confounded by ambient or personal O_3 or other pollutant exposures in the 96 and 72 hours before the pre-exposure visit. Further, these MOSES 1 O_3 effects were generally not modified, blunted, or lessened by these same ambient and personal pollutant exposures. However, the reductions in markers of pulmonary function by the MOSES 1 controlled O_3 exposure were modified by ambient NO_2 and CO, and PES NO_2, with reductions observed only when these pollutant concentrations were elevated in the few hours and days before the pre-exposure visit. Increased ambient O_3 concentrations were associated with reduced HRV, with "recovery" during exposure visits. Increased ambient $PM_{2.5}$, NO_2, and CO, were associated with reduced pulmonary function, independent of the MOSES-controlled O_3 exposures. Increased pollutant concentrations were not associated with pre-exposure or pre- to post-exposure changes in other cardiopulmonary biomarkers. Future controlled exposure studies should consider the effect of ambient pollutants on pre-exposure biomarker levels and whether ambient pollutants modify any health response to a controlled pollutant exposure.

INTRODUCTION

Exposure to air pollution is a well-established risk factor for cardiovascular morbidity and mortality. However, most of the evidence supporting an association between air pollution and adverse cardiovascular effects came from studies of particulate matter (PM). More recently, increased attention has been paid to the potential cardiovascular toxicity of O_3 because several epidemiological studies have shown an association between exposure to O_3 and mortality (U.S. Environmental Protection Agency [EPA] 2019). The evidentiary basis for the current O_3 National Ambient Air Quality Standards (NAAQS) was dominated by its acute effects on the respiratory tract, and especially by (reversible) decrements in lung function.

Tropospheric O_3 is a ubiquitous air pollutant formed when nitrogen oxides and volatile organic compounds react in the atmosphere in the presence of sunlight. O_3 is a prototypic oxidant gas that reacts with constituents of the fluid of the respiratory-tract lining to generate oxidized lipid derivatives that can cause local oxidative stress. Pathways by which O_3 could cause cardiovascular dysfunction include alterations in autonomic balance, systemic inflammation, and oxidative stress. These initial responses could lead ultimately to arrhythmias, endothelial dysfunction, acute arterial vasoconstriction, and procoagulant activity, as well as acute cardio- and cerebrovascular events (e.g., myocardial infarction, stroke, and heart failure exacerbation) and/or mortality.

There is some evidence of adverse health effects associated with chronic exposure to ambient O_3, including reduced lung growth and risk of asthma development (Frischer et al. 1999; Islam et al. 2008; McConnell et al. 2002), and increased risk of mortality from cardiovascular disease or events. For example, using data from the American Cancer Society Cancer Prevention Study II and ambient air pollutant concentrations measured in 96 United States metropolitan areas, Jerrett and colleagues reported a 1.1% increase in risk of death due to cardiovascular causes (95% CI, 0.3% to 2.6%) and a 2.9% increase in the risk of death due to respiratory causes (95% CI, 1.0% to 4.8%) associated with each 10-ppb increase in 2-year average O_3 concentration (Jerrett et al. 2009). Buteau and colleagues reported similar findings (Buteau et al. 2018). Among African American women in 56 metropolitan areas in the United States, long-term O_3 exposure was associated with an increased risk of diabetes (Jerrett et al. 2017) and hypertension (Coogan et al. 2017).

However, the evidence for an acute association between cardiovascular morbidity and mortality and increases in ambient O_3 levels in the previous few hours and days is mixed. In Stockholm County, Sweden, Raza and colleagues reported 0.7% (95% CI, 0.1% to 1.3%) and 1.2% (95% CI, 0.6% to 1.8%) higher risks of cardiovascular mortality associated with 10-$\mu g/m^3$ increases in ambient O_3 concentrations in the previous 2 days, in time-series and case-crossover studies, respectively, with larger effects among those with a prior hospitalization for acute myocardial infarction in the previous 3 years (Raza et al. 2018). Using Medicare data from 2000–2012, Di and colleagues reported a 0.51% (95% CI, 0.41% to 0.61%) increase in the daily mortality rate associated with each 10-ppb increase in warm-season O_3 concentration, after adjustment for $PM_{2.5}$ (Di et al. 2017). In a study using National Morbidity, Mortality, and Air Pollution Study (NMMAPS) data, Bell and colleagues (2004) reported a 0.64% (95% CI, 0.31% to 0.98%) increase in the risk of cardiovascular and respiratory mortality associated with each 10-ppb increase in the previous week's O_3 concentration. However, Bravo and colleagues (2016) reported no such acute association between increased O_3 concentrations and cardiovascular mortality. Others have reported increased risks of cardiovascular and/or cerebrovascular hospital and emergency-room admissions associated with increased O_3 concentrations in the previous few days (Ballester et al. 2006; Barnett et al. 2006; Chan et al. 2006; Chang et al. 2005; Halonen et al. 2010; Lee et al. 2003; Rodopoulou et al. 2014; Szyszkowicz 2008), while others have not (Corea et al. 2012; Franck et al. 2014; Fung et al. 2005; Goldberg et al. 2008; Symons et al. 2006; Tolbert et al. 2007; Zanobetti and Schwartz 2006). For example, in a recent large study of emergency department (ED) visits for cardiorespiratory outcomes and ambient air pollutants in the St. Louis, Missouri–Illinois metropolitan area, IQR increases in 8-hour maximum O_3 concentrations were associated with ED visits for respiratory disease (RR = 1.05; 95% CI, 1.02 to 1.09) and asthma/wheeze (RR = 1.07; 95% CI, 1.00 to 1.14), but not ED visits for cardiovascular disease (CVD; RR = 0.99; 95% CI, 0.95 to 1.03), ischemic heart disease (RR = 0.99; 95% CI, 0.93 to 1.05), dysrhythmia (RR = 1.00; 95% CI, 0.92 to 1.09), or congestive heart failure (RR = 1.06; 95% CI, 0.98 to 1.14). In the same study, however, ED visits for cardiovascular disease were associated with increased concentrations of organic and elemental carbon, hopanes, silicon, and iron in the previous 24 hours (Sarnat et al. 2015).

In studies of O_3 and triggering of myocardial infarction (MI), a meta-analysis of time-series and case-crossover studies reported significantly increased risks of MI associated with $PM_{2.5}$, NO_2, SO_2, and CO, but not O_3 (OR = 1.003; 95% CI, 0.997 to 1.010) (Mustafic et al. 2012). However, Evans and colleagues reported a 29% (95% CI, 0% to 63%) increase in the relative odds of a ST-elevation MI

associated with each 19.9-ppb increase in O_3 concentration in the previous hour (Evans et al. 2016). In studies of cardiac arrhythmias, acute associations (in the preceding 24 hours) between short-term O_3 exposure and ventricular arrhythmias and atrial fibrillation were reported by some (Rich et al. 2005, 2006a, 2006b), but not others (Link et al. 2013; Metzger et al. 2007; Rich et al. 2004; Vedal et al. 2004). A meta-analysis concluded that there were clear temporal relationships between stroke and several pollutants, but the weakest association was seen for O_3 (OR = 1.001; 95% CI, 1.000 to 1.002) (Shah et al. 2015). Many of these epidemiology studies are limited by common problems of exposure error and small sample sizes.

There are also mixed findings from controlled exposure and panel studies examining acute associations between short-term O_3 exposures and biomarkers of cardiovascular effects. Several previous controlled exposure studies examined whether O_3 exposures (e.g., 120–450 ppb) for 1–4 hours, some without and some with exercise (continuous or intermittent), were associated with adverse changes in cardiovascular biomarkers (Arjomandi et al. 2015; Bedi et al. 1988, 1989; Devlin et al. 1991, 2012; Drechsler-Parks et al. 1987, 1989, 1990; Gong, et al. 1997b; Kim et al. 2011; Lanzinger et al. 2014; Sivagangabalan et al. 2011; Thompson et al. 2010), while others found no such associations (Barath et al. 2013; Frampton et al. 2015; Reisenauer et al. 1988). Brook and colleagues showed that co-exposure of healthy volunteers to concentrated ambient particles and O_3 caused brachial artery vasoconstriction, without significant change in flow-mediated dilatation (FMD) (Brook et al. 2002). However, in a later study these investigators reported that O_3 alone did not have this effect (Brook et al. 2009).

Panel studies, where several biomarkers are repeatedly measured in study subjects and then related to ambient or personal measures of O_3 and other pollutants in the previous few hours and days, have also been used to examine whether ambient O_3 affects some of the same cardiovascular and pulmonary biomarkers (U.S. EPA 2013). For example, increased ambient O_3 concentrations have been associated with reductions in markers of HRV (e.g., Gold et al. 2000; Holguin et al. 2003; Jia et al. 2011; Park et al. 2005), ventricular tachycardia (e.g., Bartell et al. 2013), arterial stiffness index (e.g., Wu et al. 2010), and markers of systemic inflammation, thrombosis, and oxidative stress (e.g., Chuang et al. 2007). These studies differ from the controlled O_3 exposure studies in that they assess O_3 as part of an ambient pollutant mixture. Although they may provide a more real-world assessment of acute cardiovascular health responses to short-term O_3 exposure, O_3 effects may be difficult to disentangle from effects of other pollutants in the air pollution milieu.

The U.S. EPA's draft Integrated Science Assessment for O_3 concluded that the evidence is suggestive, but not sufficient to infer a causal association between short- and long-term O_3 exposure and cardiovascular effects (U.S. EPA 2019). This determination was largely driven by the inconsistent findings in the human clinical studies.

The Multicenter Ozone Study of oldEr Subjects (MOSES) was designed to evaluate whether short-term controlled exposure of older, healthy individuals to the peak levels of O_3 regularly experienced outdoors (120 ppb and 70 ppb) induced acute changes in cardiovascular biomarkers compared with filtered air with 0 ppb O_3. Subject recruitment started in June 2012, and the first subject was randomized on July 25, 2012. Subject recruitment ended on December 31, 2014, and testing of all 87 subjects was completed by April 30, 2015. The mean age was 59.9 ± 4.5 years, and 60% of the subjects were female, 88% were white, and 57% were glutathione S-transferase mu 1 (GSTM1) null. Mean baseline body mass index (BMI), blood pressure (BP), cholesterol (total and low-density lipoprotein), and lung function were all within the normal range. Overall, we found no significant effects of O_3 exposure on any of the primary or secondary endpoints for autonomic function, repolarization, ST segment change, or arrhythmia. O_3 exposure also did not cause significant changes in the primary endpoints for systemic inflammation (C-reactive protein [CRP]) and vascular function (systolic blood pressure [SBP] and FMD) or secondary endpoints for systemic inflammation and oxidative stress (interleukin [IL]-6, P-selectin, and 8-isoprostane). O_3 did cause a significant increase in the secondary endpoint plasma endothelin-1 (ET-1) ($P = 0.008$). A marginally significant decrease in nitrotyrosine ($P = 0.017$) may have been spurious, related to an increase in nitrotyrosine 22 hours following 0 ppb O_3, with values following 70 and 120 ppb closer to the mean. Lastly, O_3 exposure did not affect the primary prothrombotic endpoints (microparticle tissue factor activity and monocyte–platelet conjugate count) or any secondary markers of prothrombotic vascular status (platelet activation, circulating microparticles, von Willebrand factor [vWF], or fibrinogen).

Although our hypothesis focused on possible acute cardiovascular effects of exposure to low levels of O_3, we recognized that the initial effects of inhaled O_3 involve the lower airways. Therefore, we looked for: (1) changes in lung function, which are known to occur during exposure to O_3 and are maximal at the end of exposure; and (2) markers of airway injury and inflammation. We found an increase in FVC and FEV_1 after exposure to 0 ppb O_3, likely due to the

effects of exercise. The FEV_1 increased significantly 15 minutes after 0 ppb exposure (85 mL; 95% CI, 64 to 106; $P < 0.001$), and remained significantly increased from pre-exposure at 22 hours (45 mL; 95% CI, 26 to 64; $P < 0.001$). The increase in FVC followed a similar pattern. The increase in FEV_1 and FVC were attenuated in a dose–response manner by exposure to 70 and 120 ppb O_3. We also observed a significant O_3-induced increase in the percentage of sputum PMN 22 hours after exposure at 120 ppb compared with 0 ppb exposure ($P = 0.003$). Plasma club cell protein 16 (CC16) also increased significantly after exposure to 120 ppb ($P < 0.001$). Sputum IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) concentrations were not significantly different after O_3 exposure. We found no significant interactions with sex, age, or GSTM1 status regarding the effect of O_3 on lung function, percentage of sputum PMN, or plasma CC16.

These effects were seen at all three clinical sites. A complete description of MOSES, including its protocol and findings, is provided in the final report of MOSES 1 (Frampton et al. 2017) and elsewhere (Arjomandi et al. 2018; Rich et al. 2018).

In contrast with some previous controlled O_3 exposure studies (described above), where the exposure concentration levels were substantially higher than ambient levels occurring at the same time as the trial, the 120 ppb and 70 ppb O_3 concentrations used in MOSES may have been similar to or slightly higher than ambient O_3 levels at each MOSES center (i.e., Chapel Hill, North Carolina; Rochester, New York; and San Francisco, California) during the study period. Thus, ambient O_3 and other pollutant exposures experienced by the study subjects before and during the study may have independently affected the study biomarkers and/or modified any biomarker responses to the controlled O_3 exposures. Thus, MOSES 2 assessed whether personal pollutant exposures to O_3 and NO_2 or ambient air pollutant concentrations in the 72 and 96 hours, respectively, before the pre-exposure visit confounded or modified the results of MOSES 1. Further, based on the epidemiologic panel studies described above that showed effects of ambient O_3 concentrations on cardiovascular biomarkers, we assessed whether these same personal and ambient pollutant exposures independently affected the pre-exposure biomarkers levels and/or the pre- to post-exposure change in each biomarker.

HYPOTHESES AND SPECIFIC AIMS

In MOSES 1, we hypothesized a priori that short-term exposure to near-ambient concentrations of O_3 would induce acute cardiovascular responses through the following mechanisms: autonomic imbalance, systemic inflammation, and development of a prothrombotic vascular state. We also postulated that exposure to O_3 would induce airway inflammation, lung injury, and lung function decrements. Finally, we postulated the secondary hypotheses that O_3-induced acute cardiovascular responses would be associated with increased systemic oxidative stress and lung effects. To test these hypotheses, we designed a controlled exposure study of healthy older volunteers with the specific aim of examining whether short-term exposure to O_3 induces:

- Autonomic imbalance (HRV), repolarization abnormalities (T-wave amplitude), and evidence of myocardial ischemia (ST segment in the V5 lead);
- Systemic inflammation (CRP) and vascular dysfunction (blood pressure, flow-mediated dilatation);
- Prothrombotic vascular state (microparticle tissue factor activity and monocyte–platelet conjugate count);
- Lung function decrements (spirometry), airway inflammation (sputum PMN), systemic oxidative stress (8-isoprostane), and lung injury (CC16).

For MOSES 2, we used the biomarker data collected as part of MOSES 1, the personal exposures to O_3 and NO_2 concentrations measured for each study subject in the 72 hours before the pre-exposure visit of the MOSES study, as well as ambient air pollution, temperature, and relative humidity measurements in the 96 hours before the pre-exposure visit, which were retrieved from central monitoring sites near each clinical center. We first assessed whether these ambient and personal pollutant exposures confounded and/or modified the MOSES controlled O_3 exposure effects on pre- to post-exposure biomarker changes. Next, we used the same data in a longitudinal panel-study design and evaluated whether these same concentrations of personal and ambient O_3 and other pollutant concentrations, in the few hours and days before the pre-exposure visit, affected biomarker levels of MOSES subjects (both the pre-exposure levels and the pre- to post-exposure change) during the study. Our specific aims were as follows:

Aim 1 Determine whether increased personal O_3 and NO_2 concentrations in the 72 hours before the pre-exposure visit and increased ambient O_3 and other pollutant concentrations in the 1–96 hours before the pre-exposure visit confounded the MOSES 1 controlled O_3 exposure effects on the pre- to post-exposure difference in biomarkers.We hypothesized that the pre- to post-exposure biomarker differences associated with the controlled O_3 exposures in MOSES 1 would not be substantially changed when

controlling for PES O_3 in the previous 72 hours, PES NO_2 in the previous 72 hours, or ambient air pollutant concentration in the previous 96 hours.

Aim 2 Explore whether increased personal O_3 and NO_2 concentrations in the 72 hours before the pre-exposure visit and increased ambient O_3 and other pollutant concentrations in the 96 hours before the pre-exposure visit modified pre- to post-exposure biomarker responses to the MOSES 1 controlled O_3 exposures.

We formulated two alternative hypotheses:

1. The MOSES 1 controlled O_3 exposures would only cause adverse (i.e., increased or decreased biomarker changes as listed above) pre- to post-exposure biomarker changes when the PES O_3, PES NO_2, or ambient air pollutant concentrations in the 1 to 96 hours before the pre-exposure visit were *low* (e.g., any change in a biomarker from pre- to post-exposure would only occur if the PES O_3 concentration was in the lowest tertile of all subjects' PES O_3 concentrations). Otherwise, if the PES O_3 concentration was high (e.g., in the highest tertile of all subjects' PES O_3 concentration) it would mask or reduce any adverse effect of controlled O_3 exposures on these outcomes.
2. The MOSES controlled O_3 exposures would only cause adverse pre- to post-exposure biomarker changes when the PES O_3, PES NO_2, or ambient air pollutant concentrations in the 1 to 96 hours before the pre-exposure visit were *high* (i.e., there needs to be some already existing response to air pollution in order for the controlled O_3 exposure to have any measurable effect on pre- to post-exposure biomarker changes).

Aim 3 Determine whether increased personal O_3 and NO_2 concentrations in the 72 hours before the pre-exposure visit and increased ambient O_3 and other pollutant concentrations in the 96 hours before the pre-exposure visit were associated with changes in biomarkers measured at the pre-exposure visit. We hypothesized that increased PES and ambient pollutant concentrations in the 96 hours before the pre-exposure visit would be associated with the following adverse changes in pre-exposure biomarkers: decreased HRV, delayed repolarization, decreases in ST segments, increased SBP, decreased FMD, increased oxidative stress, increased systemic inflammation, increased thrombosis, reduced pulmonary function, increased airway inflammation, and increased lung injury.

Aim 4 Determine whether increased personal O_3 and NO_2 concentrations in the 72 hours before the pre-exposure visit and increased ambient O_3 and other pollutant concentrations in the 1–96 hours before the pre-exposure visit were associated with changes in each biomarker from pre- to post-exposure, independent of the controlled O_3 exposures. We hypothesized that, independent of the controlled O_3 exposures, increased PES or ambient pollutant concentrations in the 96 hours before the pre-exposure visit would be associated with one or more of the following adverse pre- to post-exposure changes: decreased HRV, delayed repolarization, depression in ST segments, increased SBP, decreased FMD, increased oxidative stress, increased systemic inflammation, increased prothrombotic markers, decreased pulmonary function, increased airway inflammation, and increased lung injury.

METHODS AND STUDY DESIGN

STUDY POPULATION

MOSES was conducted at three clinical centers: University of Rochester Medical Center (URMC; $n = 32$), University of North Carolina (UNC; $n = 29$), and the University of California, San Francisco (UCSF; $n = 26$). All subjects provided written, informed consent and the study was approved by institutional review boards at each center and by the U.S. EPA Human Subjects Research Review Official. Healthy volunteers, 55 to 70 years of age, were recruited by each center using local postings, advertising, and word-of-mouth contact. Details on subject inclusion and exclusion criteria are published in MOSES 1 (Frampton et al. 2017). Briefly, all were lifetime nonsmokers (less than 10 pack-years with no smoking in the previous 5 years, plasma cotinine level ≤ 3 ng/mL) with normal spirometry and screening electrocardiogram (ECG), able to complete a training exercise session at the target minute ventilation V_E; 15–17 L (body temperature and pressure, saturated [BTPS])/min/m^2 body surface area (BSA) without the heart rate exceeding 80% of predicted maximum, and without arrhythmias or ST depression on cardiac monitoring during exercise.

BRIEF DESCRIPTION OF ORIGINAL MOSES STUDY PROTOCOL

Figure 1 summarizes the original MOSES study protocol. The study was a randomized, crossover clinical study of 0, 70, and 120 ppb O_3 exposure for 3 hours, with intermittent exercise. Both the subject and study personnel, with the exception of the technician controlling the exposure, were blinded to the nature of the exposure. Core laboratories performed the analyses of specific outcomes for all

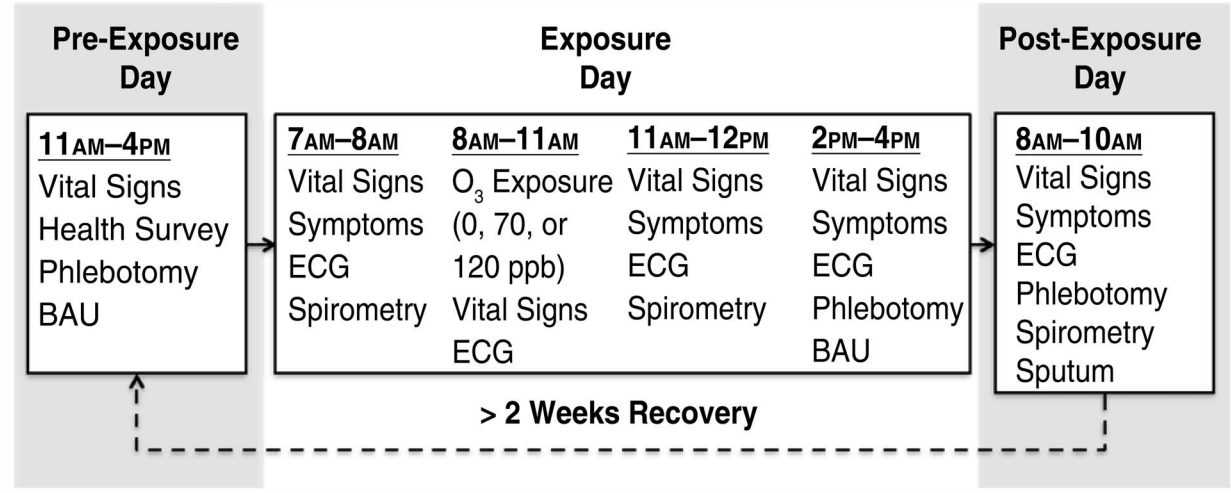


Figure 1. Measurements during 3-day visits for 3 controlled exposure sessions. Each session used a different concentration of O_3 (0, 70, or 120 ppb). The order of exposure sessions was randomly assigned to each participant.

three clinical centers; those laboratories are described in the original MOSES report (Frampton et al. 2017).

Exposure Visits Seven days prior to each exposure session the subject was contacted by either phone or email and asked about changes in health, medication use, and medication restrictions. Subjects were not studied within 6 weeks of a respiratory infection. The subject was also reminded to abstain from caffeinated beverages (e.g., coffee, tea, energy drinks, and sodas) and alcoholic beverages, starting with lunch on the day before the exposure through the post-exposure day. Each exposure session consisted of three consecutive days, as described below, with a minimum of 2 weeks between the exposure sessions.

Pre-Exposure Day The subject arrived at the laboratory or clinical research center between 11:30 am and 12:00 noon and ate a low-fat lunch (25%–30% fat). Women who were not yet postmenopausal underwent urine pregnancy testing. The following procedures were subsequently carried out: measurement of vital signs, venous blood draw (up to 30 mL), and brachial artery ultrasound for measurement of forearm FMD. A boxed dinner was provided. The subject spent the night in a nonsmoking room at a nearby hotel and was transported to and from the hotel by a hotel van or taxi.

Exposure Day The subject arrived at the laboratory or clinical research center between 7:00 am and 7:30 am, and breakfast was provided. Study procedures or measurements were carried out in the following order: BP and other vital signs were measured, symptom questionnaire answered, Holter monitor attached, HRV recorded, and spirometry test administered. The Holter ECG recording continued for 24 hours. The exposure started between 8:00 am and 8:45 am and lasted 3 hours, with intermittent exercise. Most previous controlled-exposure studies of O_3 have used exercise to increase the inhaled dose of O_3 while keeping the exposure duration manageable. The subject started with a 15-minute exercise period at the workload determined during the training visit, followed by alternating 15-minute rest and exercise periods for the duration of exposure.

Ventilation (V_E) was measured for 2 minutes twice during the first exercise period and once during the second, fourth, and sixth exercise periods; the exercise workload was adjusted as needed to achieve the targeted V_E of 15–17 L (body temperature and pressure, saturated [BTPS])/min/m^2 body surface area (BSA)without exceeding 80% of predicted maximum heart rate. BP was measured during a rest period 5 minutes before the third and fifth exercise periods. Heart rate was continuously monitored and recorded during each V_E measurement

period. During the final rest period, 10 minutes before the end of exposure, the subject filled out the symptom questionnaire. Immediately after exposure, vital signs, HRV, and spirometry were measured sequentially, and then a low-fat lunch was provided. Approximately 3 hours after the end of the exposure, the following procedures were carried out in order: HRV, venous blood draw, brachial artery ultrasound, symptom questionnaire, and vital signs. The subject went home wearing the Holter monitor at approximately 4:00–4:30 pm.

Post-Exposure Day The subject arrived at the laboratory or clinical research center at approximately 8:00 am. No breakfast was provided. However, the subject was allowed to have had breakfast if the meal had been eaten at least two hours before sputum induction (in order to avoid contamination of the sputum specimen). The following procedures were carried out: vital signs, symptom questionnaire, HRV, venous blood draw, and spirometry/sputum induction. The ECG recorder was removed, and the subject left the laboratory or clinical research center at approximately 10:30 am.

OUTCOMES ASSESSED IN MOSES 2

As described in the MOSES 1 final report, MOSES outcomes were selected based on the hypothesized mechanisms of O_3 health effects and the specific study hypotheses. For each potential mechanistic pathway for acute cardiovascular effects, we identified at least one primary outcome and several secondary outcomes. These are shown in Table 1 (modified from Sidebar 3 in the final report of MOSES 1 [Frampton et al. 2017]) and include the rationale for each group of outcomes (primary outcomes are boldfaced). Primary outcomes for each mechanistic pathway were selected based on either previous evidence in the literature that this outcome was affected by O_3 exposure (e.g., HRV) or clinical relevance (e.g., ST segment changes, BP, CRP, and FMD). The secondary outcomes were intended to help the interpretation of the results of the primary outcomes by strengthening the coherence of the findings. All outcomes were measured both before and after each exposure with the exception of sputum PMN %, which was measured only after exposure.

A complete list of all outcomes and endpoints assessed during MOSES 1 can be found in the Appendix to the Statistical Analysis Plan (found in Additional Materials 5) of the final report of MOSES 1 (Frampton et al. 2017).

All primary outcomes from MOSES 1, as well as those secondary markers for which the controlled O_3 exposures caused a change from pre- to post-exposure in MOSES 1, were included for analyses in MOSES 2 (here called "primary markers"). Furthermore, for those outcome groups, as outlined in Table 3 of the final report of MOSES 1 (Frampton et al. 2017), where the primary outcome exhibited an aim-specific significant association, we included the secondary outcomes for that outcome group in the aim-specific statistical analyses as well. Details of the methods for measurement of each biomarker are described in the final report of MOSES 1 (Frampton et al. 2017).

EXPOSURES ASSESSED IN MOSES 2

Measurement of Personal Exposure to O_3 and NO_2

Personal Exposure Sampler Personal exposure to O_3 and NO_2 during the ~72 hours preceding the pre-exposure visit were measured using a passive PES. PES components were obtained from Ogawa & Company (Pompano Beach, Florida) and assembled at each center. The shelf life of the filter used to collect the two pollutants is limited to approximately one year. Therefore, care was taken to track the dates for when the filters were received, when the PES were assembled and distributed to a subject, and when the filters were shipped to the analytic lab. The PES consisted of a small plastic reusable cylinder with two diffusion endcaps containing a glass-fiber filter coated with a nitrite-based solution for measuring O_3 and a cellulose-fiber filter coated with triethanolamine for measuring NO_2. Assembled samplers were stored in an airtight brown vial in a resealable bag at 4°C before use. Blank samplers were prepared together with the field samplers and stored together.

At the end of the training visit and at each of the first two post-exposure visits, the subject was given a PES with written instructions to store the PES in the refrigerator and start wearing it at noon of the third day before the (next) pre-exposure visit. Subjects were telephoned 3 to 4 days prior to each exposure visit and reminded to start wearing the PES. When the subject arrived at the laboratory or clinical research center, the date and time the subject started wearing the PES was recorded from the time–activity diary, and the PES was removed from the subject's clothing and disassembled. The two filters were placed in individual shipping vials and stored in the refrigerator. The storage vial containing a sampler to be used as a blank was kept closed at room temperature for three days to simulate the temperature for the active samplers while in use. The exposed and blank filters were refrigerated for up to 3 months before shipping to Research Triangle Institute, Research Triangle Park, North Carolina, for analysis. Each shipped batch of exposed filters included at least one blank.

Table 1. Primary **(boldfaced)** and Secondary Outcomes Assessed in MOSES*

1. Markers of autonomic balance (heart rate variability [HRV]), repolarization, and cardiac arrhythmia, from the ECG (Holter) recordings (averaged over 5 minutes or 24 hours).
    - Heart rate (HR) and HRV parameters: Measured in frequency and time domains, HR and HRV reflect influences of the autonomic nervous system on the heart. Reductions in parasympathetic tone lead to decreases in HRV, which are associated with heart disease and increased cardiovascular risk.
        (a) **HF** (high frequency power, 0.15–0.40 Hz), **LF** (low frequency power, 0.04–0.15 Hz), the LF/HF ratio, HR (calculated from the normal-to-normal sinus beat intervals [NN]), SDNN (standard deviation of the NN intervals), RMSSD (root mean square of successive differences in the NN intervals): 5-minute averages
        (b) **RMSSD**, HR, SDNN, HF, LF: 24-hour averages
    - Repolarization changes: Can predispose to cardiac arrhythmias; changes in the ST-segment may reflect myocardial ischemia or alterations in repolarization.
        (a) **T-wave amplitude**: 5-minute and 24-hour averages
        (b) QTc interval: 5-minute averages
    - **ST-segment changes**, ST in leads II, V2, and **V5**: 5-minute and 24-hour averages
    - Arrhythmia: ventricular ectopy and supraventricular ectopy: 24-hour total

2. Markers of systemic inflammation, oxidative stress, and vascular function: Increased systemic inflammation is associated with vascular dysfunction and cardiac disease, and increased risk for cardiovascular events.
    - **Blood pressure** (**systolic** and diastolic)
    - **Flow-mediated dilatation (FMD)**, reactive hyperemic velocity-time integral (VTI), and brachial artery diameter (BAD)
    - **C-reactive protein (CRP)**
    - 8-Isoprostane
    - Nitrotyrosine
    - Interleukin-6 (IL-6)
    - Endothelin-1 (ET-1)
    - P-selectin

3. Blood and plasma markers of prothrombotic vascular state: Increases in blood coagulability involve clotting factors, platelet function, and prothrombotic microparticles, reflecting endothelial injury and increased cardiovascular risk.
    - **Microparticle-associated tissue factor activity**
    - Von Willebrand factor (vWF)
    - Fibrinogen
    - Markers of platelet activation
        (a) **Monocyte–platelet conjugate count**
        (b) Activated (CD62P+) platelet count
        (c) Platelet-derived (CD42b+) microparticle count
        (d) Activated platelet-derived (CD42b+ and 62P+) microparticle count
        (e) Tissue factor-expressing (CD142+) microparticle count
        (f) CD40 ligand-expressing (CD154+) microparticle count

4. Markers of airway inflammation and lung injury: sputum inflammatory cells and mediators reflect inflammation in the conducting airways of the lung; blood CC16 increases in response to airway injury.
    - Sputum polymorphonuclear leukocytes (PMN) as % of total nonepithelial cells and as count/mg sputum
    - Sputum soluble markers: IL-6, interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), and total protein
    - Plasma: club cell protein 16 (CC16)

5. Spirometric parameters of pulmonary function: forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), FEV_1/FVC, and forced expiratory flow between 25% and 75% of FVC ($FEF_{25–75}$).

*Modified from Sidebar 3 of final report of MOSES 1 (Frampton et al. 2017).

Personal Exposure Sampler Filter Analyses For the analyses, the Research Triangle Institute used the default temperature of 25°C and 70% relative humidity. Both NO_2- and O_3-exposed filters were extracted in distilled water for at least 4 hours before analyses by ion chromatography. Ion chromatography calibration standards were prepared using serial dilutions of National Institute of Standards and Technology traceable stock standards. Duplicate and spike analyses were conducted at a rate of at least 1 per batch of 25 samples. The method detection limit for NO_2 was 2.3 ppb for a 24-hour sampling period and 0.77 for a 72-hour period. The detection limits for O_3 were 2.7 ppb (24-hour sampling period) and 0.9 ppb (72-hour sampling period).

Retrieval of Air Quality and Other Environmental Data

Hourly averages of temperature, relative humidity, O_3, $PM_{2.5}$, NO_2, SO_2, and CO levels were obtained from the following monitoring stations:

- New York State Department of Environmental Conservation monitoring station N-28, at Yarmouth Road, Rochester, New York (Aerometric Information Retrieval System #360551007).
- U.S. EPA air monitoring station at Alexander Drive, Durham, North Carolina (near the U.S. EPA Research Triangle Park campus). This is a research site and the data are not reported to the U.S. EPA Air Quality System. However, the site is operated with the same protocols and quality assurance procedures required for sites operated by state and local air monitoring agencies.
- Bay Area Air Quality Management District monitoring station at Arkansas Street, San Francisco, California (ARB code 90306; Aerometric Information Retrieval System #060750005).

All reported measurements of ambient and PES pollutant concentration, temperature, and relative humidity were checked for outliers, with pollutant concentration distributions, as well as correlations and scatterplots between pollutants at each site and across sites generated. From these checked and cleaned data, we calculated hourly means for the entire study period (July 1, 2012, through April 30, 2015). Some of the PES and ambient pollutant concentrations were less than zero. However, we did not correct these, as there is some measurement error throughout the distribution (e.g., at both high and low concentrations), and we did not want to alter the distribution and thus the IQR used to scale effect estimates. These hourly values were then used in the statistical analyses described below.

STATISTICAL METHODS AND DATA ANALYSIS

We generated distributions, means, and standard deviations for PES O_3, PES NO_2, each ambient pollutant (O_3, NO_2, SO_2, CO, and $PM_{2.5}$), temperature, and relative humidity for each set of lag hours before the pre-exposure visit (0, 0–2, 0–11, 0–23, 0–47, 0–71, and 0–95), separately by clinical site. We then calculated Pearson correlation coefficients between each pair of 72-hour mean concentrations of PES O_3, PES NO_2, and ambient O_3, NO_2, SO_2, CO, $PM_{2.5}$, temperature, and relative humidity (lag hours 0–71), also separately by clinical site. The main statistical analyses were conducted in accordance with the statistical analysis plan prepared prior to model fitting. Following the protocol of previous analyses, we defined each outcome for Aims 1 and 2 as a "change in biomarker," defined as the difference between the outcome at a particular time post-exposure and the value pre-exposure. For each outcome separately, a decision was made as to whether or not to use its logarithmic transformation in the models. This decision was based on plots of the residuals versus predicted values (and Q–Q plots of the residuals) from the models described below, separately for each untransformed and log-transformed biomarker. For Aims 1 and 2, this decision was also based on plots of the outcome at each post-exposure time versus the same outcome at pre-exposure, separately for both the untransformed and log-transformed scales. For variables that were log-transformed, the "change in biomarker" was defined as the difference in the log-transformed values.

Aim 1 The primary analysis for Aim 1 was a linear mixed-effects model as shown in Model 1:

$$\textbf{Model 1}: Y_{ijkt} = \mu + \tau_k + \beta x_{ijk} + \alpha_j + \beta_{ij} + \theta_t + \varepsilon_{ijkt}$$

where y_{ijkt} is the change in the outcome measure (post-exposure minus pre-exposure biomarker level) for subject i at site j at controlled O_3 concentration k at time t, μ is the intercept, τ_k is the effect of controlled O_3 exposure, β is the slope for PES O_3 concentration x for subject i in site j prior to exposure visit k (or PES NO_2 exposure, or exposure to the 1-, 3-, 12-, 24-, 48-, 72-, and 96-hour average ambient O_3, $PM_{2.5}$, CO, NO_2, and SO_2 concentrations, in separate models), α_j is the effect of site j, β_{ij} is the effect of subject i in site j, θ_t is the effect of time t, and ε is the corresponding random error, assumed to be independent and identically distributed (*iid*) Gaussian with mean 0. The controlled O_3 effect was modeled using two dummy variables to distinguish between the three levels, and both the site effect and time effect were also vectors of two dummy variables. The subject effect, β_{ij} was modeled as *iid* Gaussian with mean 0. Although not shown in Model 1 (above), we controlled for day of the week (with dummy variables), season (with

three dummy variables), and for both temperature and relative humidity using natural splines. Temperature and relative humidity were averaged over the same time period as the PES exposure (i.e., 72 hours before pre-exposure visit). The degrees of freedom for each natural spline were chosen as the value that minimized the Akaike Information Criteria (AIC). This is the same main effects model used in MOSES 1, with additional terms for temperature, relative humidity, and lagged pollutant concentration.

Model assumptions were again checked for all primary models, including examinations of plots of residuals versus predicted values, and Q–Q plots of the residuals. The 72-hour PES O_3 exposure was then replaced with the 72-hour PES NO_2 exposure, or each of the 1-, 3-, 12-, 24-, 48-, 72-, and 96-hour average ambient O_3, $PM_{2.5}$, CO, NO_2, and SO_2 concentrations, in separate models. For the analyses of FMD, there were at most 261 biomarker measurements (87 subjects × 3 visits/subject × 1 pre- to post-exposure change). For those analyses involving outcomes measured in blood (systemic inflammation, systemic oxidative stress, and prothrombotic) or via spirometry, there were 522 possible biomarker measurements (87 subjects × 3 visits/subject × 2 pre- to post-exposure changes). For those outcomes measured via Holter monitor (HRV, repolarization, and ST segment) or blood pressure measurements there were 783 possible biomarker measurements (87 subjects × 3 visits/subject × 3 pre- to post-exposure changes). From Model 1, we present the effect of the 70-ppb and 120-ppb controlled O_3 exposures (τ_k; main effect estimates from MOSES 1), after adjustment of each of the PES and ambient pollutants.

Aim 2 The model as described for Aim 1 — the same model used in MOSES 1 — was refitted for Aim 2 with two differences. First, PES O_3 exposure was categorized into tertiles. We then included the interaction between PES O_3 exposure tertiles and controlled O_3 exposure in the model. A second model was then fitted, replacing tertiles of PES O_3 exposure with tertiles of PES NO_2 exposure. This model was also re-run replacing PES tertiles with tertiles of the selected ambient O_3, $PM_{2.5}$, CO, SO_2, and NO_2 concentrations. These interaction terms allowed us to evaluate whether PES exposure or ambient pollutant concentrations modified the effect of controlled O_3 on biomarker changes. As in the final report of MOSES 1 (Frampton et al. 2017), there were at most 87 subjects or observations available for analysis.

Aim 3 The primary analysis for Aim 3 was a linear mixed-effects model, (i.e., same model used in MOSES 1) in which each subject contributed three observations (indexed by k), as shown in Model 2:

$$\textbf{Model 2 :} Y_{ijk} = \mu + \beta x_{ijk} + \alpha_j + \beta_{ij} + \theta_t + \varepsilon_{ijk}$$

where y_{ijk} is the pre-exposure biomarker level for subject i at site j prior to exposure visit k, μ is the intercept, β is the slope for PES O_3 concentration x for subject i in site j prior to exposure visit k (or, in separate models, PES NO_2 exposure or ambient exposure), α_j is the effect of site j, β_{ij} is the effect of subject i in site j, θ_t is the effect of time t, and ε_{ijk} is the corresponding random error, assumed to be iid Gaussian with mean 0. The site effect and time effect were modeled using two dummy variables each to distinguish between the three levels of site and time. The subject effect, β_{ij}, was modeled as iid Gaussian with mean 0. Although not shown in Model 2 (above), we controlled for day of the week (with dummy variables), season (with three dummy variables), time of day (time of pre- and post-exposure measurements), and for both temperature and relative humidity using natural splines. Temperature and relative humidity were averaged over the same time period as the PES exposure (i.e., 72 hours before pre-exposure visit). The degrees of freedom for each natural spline were chosen as the value that minimized the AIC. Model assumptions were checked for all primary models, including examinations of plots of residuals versus predicted values, and Q–Q plots of the residuals. The 72-hour PES O_3 exposure was then replaced with the 72-hour PES NO_2 exposure, or each of the 1-, 3-, 12-, 24-, 48-, 72-, and 96-hour average ambient O_3, $PM_{2.5}$, CO, NO_2, and SO_2 concentrations, in separate models. For these analyses, there were at most 261 biomarker measurements available for analyses (87 subjects × 3 visits/subject × 1 pre-exposure measurement).

Aim 4 For Aim 4, we used the same analyses as those in Aim 1, but present the difference in the pre- to post-exposure biomarker change associated with each increase in PES or ambient pollutant concentration (β from Model 1). Again, this was the same linear mixed-effects model used in MOSES 1, but now including temperature, relative humidity, and lagged pollutant concentrations as well. For Aim 4, we also included two pollutants in the same model as described above to determine if a difference in the pre- to post-exposure biomarker change associated with pollutant A was independent of pollutant B (e.g., was the pre- to post-exposure change in HF associated with increased O_3 concentration independent of ambient NO_2?). As in Aim 1, there were at most 261 biomarker measurements for the FMD analyses (87 subjects × 3 visits/subject × 1 pre- to post-exposure change). For those analyses involving outcomes measured in blood (systemic inflammation, systemic

oxidative stress, and prothrombotic) or via spirometry, there were 522 possible biomarker measurements (87 subjects × 3 visits/subject × 2 pre- to post-exposure changes), whereas there were 783 possible biomarker measurements for those outcomes measured via Holter monitor (HRV, repolarization, and ST segment) or blood pressure measurements (87 subjects × 3 visits/subject × 3 pre- to post-exposure changes).

Last, for each biomarker–ambient pollutant combination in Aim 4 (e.g., RMSSD and ambient O_3), we then determined which time period (1, 3, 12, 24, 48, 72, or 96 hours) had the largest parameter estimate per IQR increase in concentration (i.e., difference in the pre- to post-exposure biomarker change associated with each IQR increase in PES or ambient pollutant concentration [β from Model 2]). We then used that biomarker–pollutant lag time in the Aim 2 analyses described above.

Sensitivity Analyses For those biomarkers that were log-transformed in our MOSES 2 analyses, but not log-transformed in the MOSES 1 analyses (Frampton et al. 2017), we re-ran our Aim 3 models without log-transforming these outcomes, and compared effect estimates and inference made from the models. Second, we refit our Aim 3 models for ambient O_3, $PM_{2.5}$, CO, and NO_2 without including relative humidity. This increased the sample size, since some observations were not included due to missing relative humidity data. We then compared results and inference from these refitted Aim 3 models to those from the Aim 3 main analyses.

After comparing the effect sizes in our Aim 1 analyses to those from MOSES 1 (Frampton et al. 2017), we then focused on interpreting our Aim 2, 3, and 4 results. In interpreting the results for each outcome group (e.g., HRV, repolarization, pulmonary function, and prothrombotic vascular state) and Aim, we considered consistency across primary and secondary endpoints, direction of change consistent with that hypothesized, concentration–response relationships, and plausibility. As in MOSES 1, $\alpha = 0.01$ defined statistical significance, with $0.05 > \alpha > 0.01$ indicating marginal significance. However, although an individual effect estimate for a biomarker and pollutant may have been statistically significant, the considerations listed above were necessary to make a conclusion of effect modification of the controlled O_3 exposure effect on the pre- to post-exposure change in a biomarker (Aim 2), a conclusion of an association between a pollutant and a pre-exposure outcome group (Aim 3), or a conclusion of an association between a pollutant and a pre- to post-exposure change in an outcome group (Aim 4).

RESULTS

STUDY POPULATION

Characteristics of study subjects were previously described (Arjomandi et al. 2018; Balmes et al. 2019; Frampton et al. 2017; Rich et al. 2018). Briefly, across all sites, subjects were predominantly female (60%) and white (88%), with a mean (± standard deviation [SD]) age of 59.9 ± 4.5 years (Table 2). Subjects were generally similar across sites. However, at UNC, subjects were either African American (14%) or white (86%); while at UCSF, subjects were Asian (8%), white (88%), or Hawaiian (4%); and at URMC they were African American (3%), American Indian (3%), white (87%), or of unknown race (7%). Although there were no differences in systolic blood pressure, mean (± SD) diastolic blood pressure was higher at UNC (76.1 ± 7.8) than at UCSF (73.7 ± 10.7) and at URMC (69.0 ± 7.5) (Table 2).

AMBIENT AND PERSONAL MEASURES OF AIR POLLUTION

Distributions, means, and standard deviations of ambient O_3, NO_2, SO_2, CO, and $PM_{2.5}$, as well as temperature and relative humidity, by lag hours (0, 0–2, 0–11, 0–23, 0–47, 0–71, and 0–95) and clinical site, are shown in Table 3. Ambient O_3 concentrations were generally highest at UNC, followed by URMC, and lowest at UCSF. However, ambient NO_2 and CO concentrations were substantially higher at UCSF than at UNC or URMC. Although SO_2 concentrations were not available at the UCSF site, they were substantially higher at URMC than at UNC, whereas ambient $PM_{2.5}$ concentrations were generally highest at UCSF and lowest at URMC. As shown in Table 4, PES O_3 concentrations were similar for UCSF and UNC subjects, but both were lower than those for URMC subjects. In contrast, PES NO_2 concentrations were highest for UCSF subjects with slightly lower concentrations for URMC subjects. However, UNC subjects' concentrations were substantially lower.

Across all clinical sites, there were minimal correlations between 72-hour mean concentrations of PES O_3 and ambient O_3 (r's = 0.12 to 0.27) and PES NO_2 and ambient NO_2 (r's = 0.00 to 0.04) (Table 5). However, there were differences by site. At UCSF, most pollutants (both PES and ambient) were minimally correlated ($r < 0.40$), with the exception of ambient O_3 and CO ($r = -0.58$), ambient O_3 and NO_2 ($r = -0.66$), ambient $PM_{2.5}$ and NO_2 ($r = 0.55$), and ambient $PM_{2.5}$ and CO ($r = 0.55$), which were moderately correlated. Ambient CO and NO_2 were highly correlated ($r = 0.93$) (Table 5). However, at UNC, there were only minimal correlations between ambient and PES pollutants,

Table 2. Characteristics of MOSES Subjects by Clinical Site

	URMC (N = 32)	UNC (N = 29)	UCSF (N = 26)	Overall (N = 87)	P value
Gender					0.236
Male	12 (38%)	9 (31%)	14 (54%)	35 (40%)	
Female	20 (63%)	20 (69%)	12 (46%)	52 (60%)	
Race					0.038
American Indian	1 (3%)	0 (0%)	0 (0%)	1 (1%)	
Asian	0 (0%)	0 (0%)	2 (8%)	2 (2%)	
African American	1 (3%)	4 (14%)	0 (0%)	5 (6%)	
White	28 (87%)	25 (86%)	23 (88%)	76 (88%)	
Hawaiian	0 (0%)	0 (0%)	1 (4%)	1 (1%)	
Unknown	2 (7%)	0 (0%)	0 (0%)	1 (1%)	
GSTM1					0.632
Wild type	15 (47%)	13 (45%)	9 (35%)	37 (43%)	
Null	17 (53%)	16 (55%)	17 (65%)	50 (57%)	
Age (yrs)	59.1 ± 3.8	60.4 ± 5.1	60.3 ± 4.7	59.9 ± 4.5	0.444
Body mass index (kg/m^2)	25.0 ± 2.4	24.8 ± 3.7	24.8 ± 3.6	24.9 ± 3.2	0.948
Systolic BP (mmHg)	122.4 ± 11.4	120.4 ± 9.7	122.2 ± 12.8	121.7 ± 11.2	0.750
Diastolic BP (mmHg)	69.0 ± 7.5	76.1 ± 7.8	73.7 ± 10.7	72.8 ± 9.1	0.007
Heart rate (beats/min)	65.8 ± 11.4	63.9 ± 9.9	65.3 ± 10.1	65.0 ± 10.4	0.772
Cholesterol total (mg/dL)	208.3 ± 34.7	215.3 ± 30.7	215.8 ± 47.5	212.9 ± 37.6	0.696
LDL calc (mg/dL)	118.4 ± 30.0	119.6 ± 29.2	123.7 ± 41.8	120.4 ± 33.4	0.832
% predicted FEV_1	104.0 ± 12.8	102.4 ± 13.9	102.6 ± 12.9	103.0 ± 13.1	0.867
FEV_1 (L)	3.06 ± 0.65	2.89 ± 0.59	3.24 ± 0.73	3.06 ± 0.66	0.144
FVC (L)	3.96 ± 0.89	3.76 ± 0.79	4.24 ± 0.97	3.98 ± 0.89	0.131

except for a moderate correlation between ambient NO_2 and CO (r = 0.59). At URMC, there were again minimal correlations between all pollutants, except for ambient NO_2 and CO (r = 0.60), ambient $PM_{2.5}$ and CO (r = 0.47), and ambient $PM_{2.5}$ and NO_2 (r = 0.44).

AIM 1

To further exclude confounding of the controlled O_3 exposure effects reported in MOSES 1 by prior ambient pollutant exposures, we re-ran the analytic models from MOSES 1 for all outcome variables, adding to the models the PES and ambient pollutant concentrations at all lag averaging times individually. For each outcome, there were 37 models adjusting for PES O_3, PES NO_2, or ambient O_3, $PM_{2.5}$, CO, NO_2, SO_2 in the 1, 3, 12, 24, 48, 72, and 96 hours before the pre-exposure visit. For those outcomes that were log-transformed in MOSES 2, but not in MOSES 1, we present analyses of both outcomes. Overall, addition of the ambient pollutants to the model did not substantially alter the MOSES 1 results (Additional Materials 1, Tables 1 and 2). For example, in MOSES 1, O_3 exposure caused a significant decrease (P = 0.003) in FEV_1 (120 ppb: −0.033 L, 95% CI, −0.051 to 0.014; 70 ppb: −0.015 L, 95% CI, −0.033 to −0.004). When adjusting for ambient O_3 in the 24 hours before the pre-exposure visit, O_3 exposure still caused a significant decrease (P = 0.002) in FEV_1 (120 ppb: −0.04 L, 95% CI, −0.01 to −0.02; 70 ppb: −0.01 L, 95% CI, −0.04 to 0.01). Almost all of the 37 models for FEV_1 followed this pattern. However, when adjusting for ambient NO_2 in the 1 hour before the pre-exposure visit, O_3 exposure did not cause a significant decrease (P = 0.088) in FEV_1, and the effect estimates were somewhat reduced (120 ppb: −0.02 L, 95% CI, −0.04 to 0.00; 70 ppb: −0.01 L, 95% CI,= −0.03 to 0.01). Most cardiovascular, oxidative stress, and coagulation outcomes showed no effects of the

Table 3. Distribution of Ambient Pollutant Concentrations and Meteorology Characteristics During the Study Period, by MOSES Clinical Site

Parameter / Clinical Site	Lag Hours	*N*	Mean	Standard Deviation	Minimum[a]	5th	25th	50th	75th	95th	Maximum
O_3 (ppb)											
UCSF	0	70	26.16	9.10	3.60	10.90	20.70	25.85	32.00	41.20	46.90
	0–2	67	23.95	8.92	3.53	8.17	19.07	24.07	29.00	39.20	45.90
	0–11	71	20.15	10.37	1.99	3.81	11.94	21.65	26.79	36.75	44.26
	0–23	73	23.10	8.85	4.38	8.94	17.26	23.25	28.56	37.57	44.87
	0–47	73	23.48	8.17	7.04	8.99	17.73	24.22	28.07	36.95	44.12
	0–71	73	23.16	7.78	7.09	8.40	18.46	23.91	27.51	37.03	42.08
	0–95	73	22.88	7.46	6.77	8.48	19.19	22.72	26.73	35.55	39.29
UNC	0	72	35.54	12.63	−0.10	12.10	26.70	35.60	42.90	55.10	64.00
	0–2	69	32.01	12.23	−0.17	9.73	24.80	31.20	39.30	52.33	63.20
	0–11	69	21.44	10.61	−0.25	3.35	13.99	19.59	30.24	37.13	41.83
	0–23	70	26.58	10.15	−0.23	12.33	19.61	26.12	35.02	42.69	45.88
	0–47	71	27.82	9.45	−0.22	10.98	21.25	29.58	34.64	42.48	45.04
	0–71	70	28.21	9.31	−0.21	14.25	21.85	28.64	35.97	41.22	45.74
	0–95	70	28.64	8.44	−0.21	16.80	22.25	29.74	34.19	40.41	48.17
URMC	0	86	32.38	12.37	5.00	12.00	23.00	33.50	39.00	51.00	74.00
	0–2	80	29.09	11.26	4.00	9.17	21.00	30.67	36.33	44.83	65.33
	0–11	92	22.87	9.72	1.20	8.70	14.21	23.25	29.79	39.33	44.75
	0–23	92	27.00	9.06	10.55	13.42	20.65	26.02	32.90	43.46	52.42
	0–47	92	27.20	7.79	10.76	16.54	21.78	26.44	32.23	40.00	50.52
	0–71	92	26.63	7.35	9.67	15.32	21.11	26.22	31.33	38.40	45.83
	0–95	93	26.48	7.02	9.40	15.65	21.81	25.59	31.23	37.78	45.34
NO_2 (ppb)											
UCSF	0	70	11.31	9.48	3.50	3.60	4.70	7.35	14.80	29.90	45.30
	0–2	67	12.57	9.24	3.40	4.13	5.57	8.23	17.90	31.53	39.60
	0–11	71	14.69	10.44	3.90	4.72	6.64	9.79	19.98	36.70	40.13
	0–23	73	11.96	9.21	3.43	3.99	4.83	8.85	15.53	31.92	38.43
	0–47	73	10.85	8.56	3.27	3.45	4.29	7.72	14.55	30.12	38.17
	0–71	73	10.88	8.34	3.15	3.56	4.42	7.35	14.16	29.17	37.35
	0–95	73	11.26	8.33	3.37	3.73	5.49	8.00	14.07	31.52	38.40
UNC	0	66	4.91	5.64	−0.10	1.40	2.40	3.25	5.70	10.40	35.20
	0–2	64	5.32	4.29	−0.23	1.70	2.82	4.12	6.47	14.03	23.93
	0–11	64	7.22	5.65	0.00	2.29	3.58	4.79	8.80	20.00	25.92
	0–23	64	6.45	4.61	0.00	1.94	3.58	4.64	7.47	15.38	22.26
	0–47	64	6.10	3.33	0.04	2.15	3.65	5.44	7.56	13.90	15.30
	0–71	63	6.15	3.41	0.03	2.30	3.90	5.37	7.96	11.39	20.37
	0–95	62	6.07	2.97	0.57	2.67	4.53	5.25	7.52	11.18	16.80
URMC	0	83	4.61	3.66	−2.20	−0.30	2.40	4.00	7.10	10.40	20.50
	0–2	83	5.94	4.17	−1.90	0.20	3.20	4.80	7.53	14.43	19.40
	0–11	86	8.15	5.19	−1.53	2.54	4.58	7.05	10.23	18.03	26.13
	0–23	87	6.72	3.88	−0.72	2.00	3.86	6.07	9.12	13.72	17.59
	0–47	87	6.31	3.44	0.70	1.64	3.78	5.65	8.00	12.86	17.58
	0–71	87	6.48	3.39	1.47	2.04	4.08	6.04	8.14	12.22	18.52
	0–95	87	6.45	3.09	1.36	2.07	4.09	6.13	7.96	13.46	18.11

Table continues next page

[a] Concentrations below 0 were unchanged, so that the distribution and interquartile range used to scale effect estimates were unaltered.

Table 3 (*Continued*). Distribution of Ambient Pollutant Concentrations and Meteorology Characteristics During the Study Period, by MOSES Clinical Site

Parameter / Clinical Site	Lag Hours	*N*	Mean	Standard Deviation	Minimum[a]	5th	25th	50th	75th	95th	Maximum
SO_2 (ppb)											
UCSF	0 0–2 0–11 0–23 0–47 0–71 0–95	No SO_2 data for UCSF									
UNC	0	60	0.46	0.76	−0.30	−0.10	0.00	0.20	0.65	1.95	4.20
	0–2	59	0.62	1.18	−0.27	−0.13	0.00	0.23	0.70	3.47	7.30
	0–11	57	0.36	0.57	−0.57	−0.28	−0.05	0.19	0.53	1.67	1.79
	0–23	58	0.35	0.72	−0.61	−0.26	−0.04	0.18	0.45	1.63	4.16
	0–47	59	0.34	0.74	−0.71	−0.45	−0.05	0.18	0.41	1.71	3.92
	0–71	59	0.30	0.55	−0.65	−0.30	−0.03	0.14	0.43	1.35	2.50
	0–95	58	0.29	0.46	−0.54	−0.20	0.00	0.19	0.44	1.45	1.75
URMC	0	88	1.05	1.16	0.09	0.14	0.41	0.74	1.35	3.02	8.53
	0–2	88	1.15	1.53	0.13	0.18	0.45	0.70	1.28	3.16	10.12
	0–11	92	0.97	1.06	0.11	0.15	0.41	0.66	1.11	3.34	5.72
	0–23	93	1.04	0.93	0.11	0.15	0.43	0.69	1.33	3.46	4.84
	0–47	93	1.03	0.71	0.12	0.22	0.51	0.89	1.31	2.50	3.61
	0–71	93	1.01	0.59	0.14	0.24	0.53	0.90	1.32	2.13	2.93
	0–95	93	1.02	0.55	0.14	0.29	0.59	0.92	1.41	1.98	2.63
CO (ppm)											
UCSF	0	70	0.35	0.11	0.22	0.23	0.27	0.33	0.40	0.58	0.75
	0–2	67	0.37	0.12	0.22	0.25	0.28	0.33	0.41	0.57	0.75
	0–11	71	0.42	0.17	0.21	0.25	0.29	0.36	0.50	0.78	0.86
	0–23	73	0.39	0.15	0.20	0.23	0.28	0.34	0.44	0.70	0.78
	0–47	73	0.37	0.14	0.20	0.22	0.28	0.32	0.42	0.67	0.75
	0–71	73	0.37	0.13	0.20	0.22	0.27	0.32	0.44	0.65	0.72
	0–95	73	0.37	0.13	0.20	0.22	0.27	0.32	0.43	0.67	0.74
UNC	0	70	0.16	0.06	−0.05	0.12	0.14	0.16	0.18	0.22	0.39
	0–2	68	0.17	0.07	−0.05	0.11	0.14	0.17	0.19	0.28	0.50
	0–11	69	0.21	0.09	0.06	0.12	0.16	0.18	0.23	0.43	0.52
	0–23	69	0.19	0.07	0.05	0.12	0.15	0.18	0.20	0.35	0.40
	0–47	69	0.19	0.05	0.06	0.12	0.16	0.18	0.21	0.25	0.34
	0–71	69	0.19	0.05	0.08	0.12	0.16	0.19	0.21	0.26	0.39
	0–95	68	0.19	0.04	0.10	0.12	0.17	0.19	0.21	0.25	0.38
URMC	0	88	0.19	0.07	0.00	0.11	0.15	0.18	0.22	0.34	0.41
	0–2	88	0.20	0.07	0.00	0.12	0.16	0.19	0.23	0.33	0.39
	0–11	89	0.20	0.07	0.00	0.12	0.17	0.19	0.25	0.33	0.49
	0–23	89	0.20	0.06	0.00	0.13	0.17	0.19	0.23	0.30	0.39
	0–47	89	0.20	0.05	0.01	0.11	0.17	0.19	0.22	0.27	0.43
	0–71	89	0.20	0.05	0.01	0.11	0.17	0.19	0.23	0.29	0.41
	0–95	89	0.20	0.05	0.07	0.11	0.17	0.19	0.22	0.29	0.41

Table continues next page

[a] Concentrations below 0 were unchanged, so that the distribution and interquartile range used to scale effect estimates were unaltered.

Table 3 (*Continued*). Distribution of Ambient Pollutant Concentrations and Meteorology Characteristics During the Study Period, by MOSES Clinical Site

Parameter / Clinical Site	Lag Hours	*N*	Mean	Standard Deviation	Minimum[a]	5th	25th	50th	75th	95th	Maximum
$PM_{2.5}$ (µg/m³)											
UCSF	0	69	8.93	7.12	−4.00	0.00	5.00	7.00	12.00	27.00	29.00
	0–2	66	9.26	6.73	−0.67	0.67	4.67	7.67	12.33	22.00	31.67
	0–11	72	9.39	6.77	0.42	1.58	5.04	7.56	12.38	22.00	37.92
	0–23	71	8.83	5.33	1.50	2.33	5.08	7.33	11.83	16.91	28.63
	0–47	72	8.71	4.55	1.89	3.17	5.07	7.67	11.28	18.25	25.71
	0–71	72	8.76	4.19	2.06	3.47	6.07	7.43	11.61	16.50	23.01
	0–95	72	8.80	4.29	1.81	3.05	6.10	7.71	11.02	15.86	22.33
UNC	0	66	7.13	4.65	0.80	2.20	4.40	6.05	9.50	15.60	26.90
	0–2	63	7.58	4.85	0.87	2.13	4.47	6.27	9.43	15.60	27.40
	0–11	64	8.38	4.20	1.76	2.73	5.23	7.39	10.64	16.13	20.22
	0–23	65	7.72	3.62	1.42	2.35	5.05	7.04	9.56	14.73	15.82
	0–47	65	7.99	3.68	1.08	2.94	5.55	7.48	9.81	15.08	17.04
	0–71	65	7.96	3.28	1.03	3.36	5.88	7.52	10.51	13.43	15.88
	0–95	65	8.09	3.05	1.04	3.71	6.11	8.07	10.25	13.08	15.60
URMC	0	88	6.45	5.25	−0.64	0.30	3.30	5.15	8.33	16.68	31.30
	0–2	87	6.88	5.00	−0.90	1.00	3.81	5.70	9.10	15.67	27.50
	0–11	90	6.70	3.89	0.70	1.74	3.94	5.95	8.89	14.28	22.44
	0–23	91	6.57	3.11	0.71	2.43	4.60	5.81	8.25	12.74	14.92
	0–47	91	6.72	2.70	0.73	2.54	4.86	6.16	8.24	12.14	13.95
	0–71	92	6.64	2.56	1.14	3.37	4.79	6.17	7.91	11.84	13.29
	0–95	91	6.50	2.33	0.90	3.32	4.92	6.09	7.83	10.33	13.39
Relative Humidity (%)											
UCSF	0	55	65.51	15.57	24.80	33.40	54.40	70.70	78.30	83.40	87.40
	0–2	55	67.98	14.57	31.00	41.03	55.83	71.03	79.63	86.13	89.07
	0–11	55	78.86	10.84	45.45	57.24	73.53	80.50	87.51	93.06	94.61
	0–23	55	75.51	12.09	33.28	49.79	69.56	79.58	83.86	89.33	91.92
	0–47	55	76.32	11.05	33.65	54.14	71.35	79.74	82.96	88.82	89.51
	0–71	55	76.60	10.41	33.28	56.34	72.31	79.19	83.59	88.05	88.68
	0–95	55	76.35	10.50	32.29	53.11	73.31	78.56	81.89	88.13	88.91
UNC	0	82	56.41	19.37	20.00	25.00	43.00	52.50	71.00	90.00	92.00
	0–2	80	60.73	18.16	22.33	28.00	46.67	58.00	74.17	90.50	93.00
	0–11	80	74.24	14.04	30.08	45.96	65.38	79.80	84.46	90.46	91.83
	0–23	80	66.79	15.36	32.54	39.83	57.58	69.31	79.38	86.50	92.38
	0–47	80	65.11	14.10	31.54	39.11	55.04	68.27	75.33	86.19	91.58
	0–71	80	64.53	12.26	35.82	43.94	55.50	65.73	73.01	84.80	87.40
	0–95	81	64.92	10.76	35.81	46.86	59.34	64.23	72.28	81.77	86.13
URMC	0	93	54.40	17.06	21.00	32.00	42.00	53.00	63.00	87.00	97.00
	0–2	93	58.15	16.31	25.00	34.67	47.00	56.67	66.00	90.00	97.00
	0–11	93	71.66	12.51	32.00	49.33	65.67	71.92	79.50	92.00	95.83
	0–23	93	65.85	12.85	28.71	40.00	57.13	67.17	73.88	87.50	90.92
	0–47	93	65.73	10.80	38.63	46.25	58.33	65.42	73.31	83.17	90.04
	0–71	93	65.93	9.65	46.64	49.46	60.04	65.01	73.96	81.89	87.06
	0–95	93	65.96	8.66	46.46	51.10	59.40	66.54	72.48	79.86	88.90

Table continues next page

[a] Concentrations below 0 were unchanged, so that the distribution and interquartile range used to scale effect estimates were unaltered.

Table 3 (*Continued*). Distribution of Ambient Pollutant Concentrations and Meteorology Characteristics During the Study Period, by MOSES Clinical Site

Parameter / Clinical Site	Lag Hours	*N*	Mean	Standard Deviation	Minimum[a]	5th	25th	50th	75th	95th	Maximum
Temperature (°C)											
UCSF	0	72	16.53	3.75	8.30	11.00	13.95	16.15	18.20	24.00	27.40
	0–2	72	15.90	3.72	7.33	10.13	13.05	15.63	17.72	22.13	24.77
	0–11	72	13.59	2.91	6.26	9.04	11.15	14.00	16.03	18.00	19.72
	0–23	72	14.44	2.86	7.43	9.70	12.41	14.28	16.58	19.31	22.29
	0–47	72	14.24	2.55	7.18	9.84	12.84	13.93	16.08	18.31	20.87
	0–71	72	14.15	2.40	7.36	10.21	12.72	14.04	15.69	17.93	20.88
	0–95	72	14.18	2.34	7.53	10.11	12.71	14.29	15.77	17.71	20.71
UNC	0	82	16.78	8.81	−1.50	3.60	8.70	17.35	24.10	29.90	32.10
	0–2	80	15.70	8.74	−1.87	2.22	8.25	15.67	22.88	29.12	30.60
	0–11	80	12.40	7.94	−4.62	−0.94	7.15	11.73	18.98	24.40	25.18
	0–23	80	14.15	7.62	−2.13	1.43	8.44	13.57	19.75	25.80	27.10
	0–47	80	13.96	7.54	−3.91	2.79	8.31	13.18	20.33	25.68	27.04
	0–71	80	13.75	7.61	−4.35	3.60	7.12	13.14	20.28	25.32	27.30
	0–95	81	13.39	7.70	−3.21	3.16	6.58	12.79	19.67	25.21	26.81
URMC	0	93	13.25	11.35	−11.17	−5.67	3.83	13.17	23.72	28.72	31.00
	0–2	93	12.33	11.04	−10.09	−6.19	2.50	12.22	22.15	27.46	29.96
	0–11	93	9.13	9.70	−11.24	−7.91	1.38	8.99	18.19	22.72	26.22
	0–23	93	10.42	10.17	−9.74	−7.79	2.12	9.94	20.33	24.62	28.32
	0–47	93	10.39	9.81	−6.59	−5.60	1.87	9.71	19.78	24.16	27.04
	0–2	93	10.24	9.60	−7.85	−5.12	1.91	9.59	19.76	23.40	26.59
	0–11	93	10.17	9.52	−8.23	−6.09	2.22	9.94	19.42	23.27	26.19

[a] Concentrations below 0 were unchanged, so that the distribution and interquartile range used to scale effect estimates were unaltered.

experimental O_3 exposures, as in MOSES 1. Those endpoints that were significantly changed by the MOSES 1 controlled O_3 exposures, remained statistically significant. For example, the significant increases in ET-1 and marginally significant decreases in nitrotyrosine in response to the controlled O_3 exposure, seen in the MOSES 1 analysis (Frampton et al. 2017), remained significant when adding most pollutants at different lag times. The same was true for FVC and airway injury. Thus, we did not find any evidence for confounding of the MOSES 1 results by PES or ambient pollutant concentrations.

AIM 2

Next, we examined if these same PES and ambient air pollutant concentrations in the 1 to 96 hours before the pre-exposure visit modified the effect of the MOSES controlled O_3 exposures on pre- to post-exposure changes in the primary biomarkers. We wanted to assess whether the conclusion made in the final report of MOSES 1 — of no adverse cardiovascular effects of controlled O_3 exposure — was unchanged after taking into account the PES and ambient air pollutant concentrations in the 96 hours before the pre-exposure visit. A priori, we hypothesized that the MOSES controlled O_3 exposures may only cause adverse pre- to post-exposure biomarker changes when the PES O_3, PES NO_2, or ambient air pollutant concentrations in the 1 to 96 hours before the pre-exposure visit were low (e.g., any change in a biomarker from pre- to post-exposure would only occur if the PES O_3 concentration was in the lowest tertile of all subjects' PES O_3 concentrations). Otherwise, if the PES O_3 concentration was

Table 4. Distributions of PES Measurements in the 72 Hours before the Pre-Exposure Visit, by MOSES Clinical Site[a,b]

Pollutant	Clinical Site	Subjects (*N*)	PES (*N*)	Mean	Standard Deviation	Minimum[c]	5th	25th	50th	75th	95th	Maximum
O_3 (ppb)	UCSF	26	73	3. 70	3.24	0.00	0.40	1.46	2.58	4.72	10.33	13.30
	UNC	29	85	3.83	3.65	−0.84	0.29	1.40	2.62	5.06	11.22	17.34
	URMC	32	93	3.93	4.65	−1.29	0.01	0.83	1.96	5.40	14.24	20.03
NO_2 (ppb)	UCSF	26	73	14.44	10.98	1.00	3.36	7.93	12.05	16.57	35.06	71.35
	UNC	29	85	4.00	5.85	−2.89	−1.97	0.36	2.98	4.99	14.07	29.78
	URMC	32	93	10.04	9.77	−1.89	1.70	4.54	7.19	12.40	26.46	72.36

[a] Includes data from all subjects who completed all three exposures and had a least one PES.

[b] Statistics were calculated based on the blank-corrected data.

[c] Concentrations below 0 were unchanged, so that the distribution and interquartile range used to scale effect estimates were unaltered.

Table 5. Correlation between PES (lag hours 0–71) and ambient air pollutant concentrations (lag hours 0–71), by MOSES clinical site UCSF[a], UNC[b], and URMC[c]

	PES		Ambient						
	O_3	NO_2	O_3	$PM_{2.5}$	CO	NO_2	SO_2	Temperature	Relative Humidity
PES O_3	—	−0.11	0.27	−0.02	−0.23	−0.25	—	0.19	0.20
PES NO_2	−0.10	—	−0.06	−0.09	0.05	0.04	—	0.10	−0.17
Ambient O_3	0.12	−0.09	—	−0.36	−0.58	−0.66	—	0.04	0.19
Ambient $PM_{2.5}$	0.32	−0.20	−0.20	—	0.55	0.55	—	−0.05	−0.34
Ambient CO	−0.01	−0.13	−0.12	0.25	—	0.93	—	−0.22	−0.69
Ambient NO_2	−0.03	0.00	0.01	0.17	0.59	—	—	−0.28	−0.73
Ambient SO_2	0.08	0.14	0.20	−0.02	0.03	0.06	—	—	—
Temperature	0.24	−0.17	0.03	0.19	−0.56	−0.65	−0.10	—	−0.07
Relative humidity	0.13	−0.18	−0.48	0.26	−0.14	−0.29	−0.21	0.54	—
PES O_3	—	−0.15	0.23	−0.02	−0.20	−0.31	−0.36	0.69	−0.15
PES NO_2		—	0.09	−0.01	−0.06	0.04	0.22	−0.18	−0.11
Ambient O_3			—	0.05	−0.24	−0.36	0.20	0.09	−0.57
Ambient $PM_{2.5}$				—	0.47	0.44	0.08	0.01	0.15
Ambient CO					—	0.60	0.16	−0.19	0.20
Ambient NO_2						—	0.30	−0.36	0.12
Ambient SO_2							—	−0.53	−0.25
Temperature								—	−0.08
Relative humidity									—

[a] UCSF: University of California, San Francisco, shown in white cells.

[b] UNC: University of North Carolina, shown in blue cells.

[c] URMC: University of Rochester Medical Center, shown in green cells.

high (e.g., in the highest tertile of all subjects' PES O_3 concentration), it would block or lessen any adverse effect of controlled O_3 exposures on these outcomes (Hypothesis #1). However, another scenario is also possible. It could be that the controlled O_3 exposures would only have adverse effects on pre- to post-exposure biomarker changes when the PES O_3, PES NO_2, or ambient air pollutant concentrations in 1 to 96 hours before the pre-exposure visit were in the highest tertile (i.e., there needs to be some already existing response to air pollution in order for the controlled O_3 exposure to have any measurable effect on the pre- to post-exposure biomarker changes) (Hypothesis #2). Therefore, we present results for the primary outcomes in each outcome group, with figures presented for those ambient or PES pollutants that significantly modified ($P < 0.01$) the MOSES controlled O_3 exposure/biomarker response. If the pattern of primary biomarker changes associated with the 70-ppb and 120-ppb controlled O_3 exposures within each tertile appeared consistent with Hypothesis #1 or Hypothesis #2, we then present and describe the secondary outcomes for that outcome group. Last, we then provide a summary of findings for that outcome group considering results for the primary and, if needed, secondary outcomes. The results and the Aim 2 conclusions across each outcome group are also summarized in Table 6.

Heart Rate Variability

Ambient NO_2 concentrations in the 72 hours before the pre-exposure visit significantly ($P = 0.004$) modified the pre- to post-exposure change in HF associated with the MOSES controlled O_3 exposures (Figure 2A; Additional Materials 1, Table 3; available on the HEI website). However, inconsistent with the expected direction of effect (i.e., increased air pollutant concentrations associated with decreased HF), in both the Low and High tertiles, HF increased following the 120-ppb O_3 exposure (0.543 ln[ms^2]; 95% CI, 0.089 to 0.996 and 0.551 ln[ms^2]; 95% CI, 0.099 to 1.002; respectively), compared with the 0-ppb O_3 exposure. There was no effect of the 70-ppb O_3 exposure on HF in either tertile. In contrast, in the Medium tertile, HF decreased following the 120-ppb O_3 exposure (−0.41 ln[ms^2]; 95% CI, −0.826 to 0.007), but the 70-ppb O_3 exposure had no effect.

Similarly, for LF, ambient O_3 concentrations in the 3 hours before the pre-exposure visit significantly ($P = 0.001$) modified the association between controlled O_3 exposure and LF (Figure 2B; Additional Materials 1, Table 3). In the High tertile, there was no difference in LF associated with the 70-ppb controlled O_3 exposure, but LF increased following the 120-ppb exposure (0.437 ln[ms^2]; 95% CI, 0.051 to 0.823). However, in the Medium and Low tertiles, there were both increased LF and decreased LF following the 70-ppb exposure, but no LF change following the 120-ppb O_3 exposure. CO concentrations in the 72 hours before the pre-exposure visit also significantly ($P = 0.008$) modified the LF change following the MOSES controlled O_3 exposure (Figure 2C; Additional Materials 1, Table 3). The 120-ppb and 70-ppb exposures were not associated with any significant changes in LF in the Medium or Low tertiles, but there was an exposure–response pattern in the High tertile, with a 0.394 ln(ms^2) increase in LF (95% CI, −0.027 to 0.814) following the 70-ppb controlled O_3 exposure, and an even larger increase following the 120-ppb O_3 exposure (0.551 ln[ms^2]; 95% CI, 0.154 to 0.947) (Figure 2C). However, there did not appear to be effect modification of the controlled O_3 exposure/RMSSD (24 hour; also a primary HRV marker) association by any pollutant (Additional Materials 1, Table 3).

We next examined effect modification by secondary HRV markers (RMSSD [5 min], SDNN [5 min], and LF/HF ratio [5 min]) (Additional Materials 2). Ambient CO in the previous 12 hours significantly modified the effect of the MOSES controlled O_3 exposures on ln(RMSSD) ($P = 0.005$) in a pattern consistent with Hypothesis #2 (Figure 2D; Additional Materials, Appendix A). In the High tertile, there was an exposure–response pattern with a larger increase in RMSSD following the 120-ppb exposure (0.367 ln[ms^2]; 95% CI, 0.190 to 0.543), with a smaller effect following the 70-ppb exposure (0.208 ln[ms^2]; 95% CI, 0.038 to 0.378). In the Medium and Low tertiles, there were no clear effects of either the 70- or 120-ppb exposures. Similarly, there was a similar pattern of effect modification by ambient NO_2 in the previous 12 hours, which was also consistent with Hypothesis #2 (Figure 2E; Additional Materials 2, Appendix A). However, although ambient NO_2 in the previous 72 hours significantly ($P = 0.001$) modified the effect of the MOSES controlled O_3 exposures on SDNN, the pattern was not consistent with either Hypothesis #1 or Hypothesis #2 (Figure 2F; Additional Materials 2, Appendix A). For example, in the Low tertile, the 70-ppb exposure caused a decreased SDNN (−0.163 ln[ms^2]; 95% CI, −0.332 to 0.006), while the 120-ppb exposure caused an increased SDNN (0.150 ln[ms^2]; 95% CI, −0.036 to 0.336). In contrast, in the Medium tertile, the 120-ppb O_3 exposure caused a decrease in SDNN (−0.261 ln[ms^2]; 95% CI, −0.432 to −0.089), with the 70-ppb exposure having no effect. There was no effect for either the 70-ppb or 120-ppb O_3 exposure in the High tertile. Similarly, although both ambient NO_2 in the previous 72 hours ($P = 0.003$; Figure 2G) and ambient O_3 in the previous 96 hours ($P = 0.001$; Figure 2H) significantly modified the effect of the MOSES controlled O_3 exposures

Table 6. Summary of Aim 2 Findings and Conclusions

Outcome Group	Primary Endpoints	Secondary Endpoints	Conclusion
AIM 2: Were statistically significant or marginally significant results consistent with Hypothesis #1 or #2?			
Heart rate variability	Not consistent with Hypothesis #1 or #2 HF — ambient NO_2 (prior 72 hr) LF — ambient O_3 (prior 3 hr) LF — ambient CO (prior 72 hr)	Consistent with Hypothesis #2 RMSSD (5 min) — ambient CO (prior 12 hr) RMSSD (5 min) — ambient NO_2 (prior 12 hr) Not consistent with Hypothesis #1 or #2 SDNN — ambient NO_2 (prior 72 hr) LF/HF ratio — ambient NO_2 (prior 72 hr) LF/HF ratio — ambient O_3 (prior 96 hr)	Results not consistent across heart rate variability primary and secondary markers. No effect modification
Repolarization	Not consistent with Hypothesis #1 or #2 T-wave (5 min) — ambient SO_2 (prior 72 hr) T-wave (24 hr) — ambient $PM_{2.5}$ (prior 1 hr) T-wave (24 hr) — PES NO_2 (prior 72 hr)	—	No effect modification
ST segment	None	—	No effect modification
Vascular function	None	—	No effect modification
Systemic inflammation	Not consistent with Hypothesis #1 or #2 CRP — ambient $PM_{2.5}$ (prior 3 hr)	—	No effect modification
Systemic oxidative stress	None	—	No effect modification
Prothrombotic vascular state	Consistent with Hypothesis #2 Monocyte platelet conjugate count — ambient $PM_{2.5}$ (prior 96 hr) Not consistent with Hypothesis #1 or #2 Monocyte platelet conjugate count — PES O_3 (prior 72 hr) Monocyte platelet conjugate count — ambient $PM_{2.5}$ (prior 72 hr)	—	No agreement between the two primary prothrombotic markers. No effect modification
Pulmonary function	Consistent with Hypothesis #2 FEV_1 — ambient CO (prior 3 hr) FEV_1 — PES NO_2 (prior 72 hrhr) FVC — ambient NO_2 (prior 72 hr) FVC — PES NO_2 (prior 72 hr)	No secondary markers of pulmonary function	Effect modification by ambient NO_2, CO, and PES NO_2 in the 72 hr before the pre-exposure visit, consistent with Hypothesis #2
Lung injury	None	—	No effect modification
Sputum	None	—	No effect modification

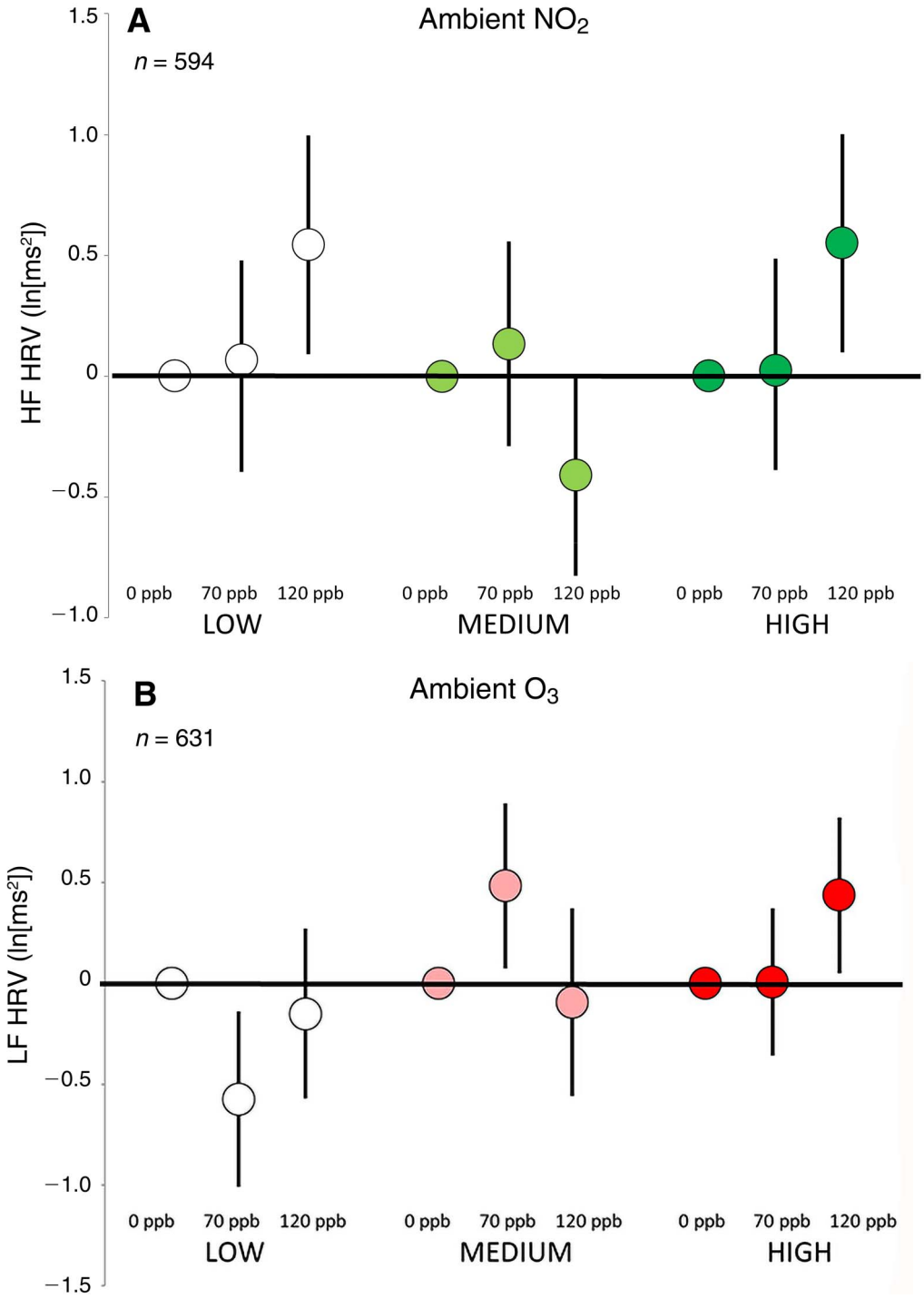


Figure 2. Change heart rate variability measures associated with each controlled O_3 exposure, by tertile of ambient pre-exposure pollutant concentration: (A) ln(ms^2) of HF HRV for NO_2 (lag hours 0–71) (test of interaction $P = 0.004$); **(B)** ln(ms^2) of LF HRV for O_3 (lag hours 0–2) (test for interaction $P = 0.001$); **(C)** ln(ms^2) of LF HRV for CO (lag hours 0–71) (test for interaction $P = 0.008$); **(D)** ln(ms) of RMSSD HRV for CO (lag hours 0–11) (test for interaction $P = 0.005$); **(E)** ln(ms) of RMSSD HRV for NO_2 (lag hours 0–11) (test for interaction $P = 0.002$); **(F)** ln(ms) of SDNN HRV for NO_2 (lag hours 0–71) (test for interaction $P = 0.001$); **(G)** LF/HF ratio for NO_2 (lag hours 0–71) (test for interaction $P = 0.003$); and **(H)** LF/HF ratio for O_3 (lag hours 0–95) (test for interaction $P = 0.001$). (*Figure continues next page.*)

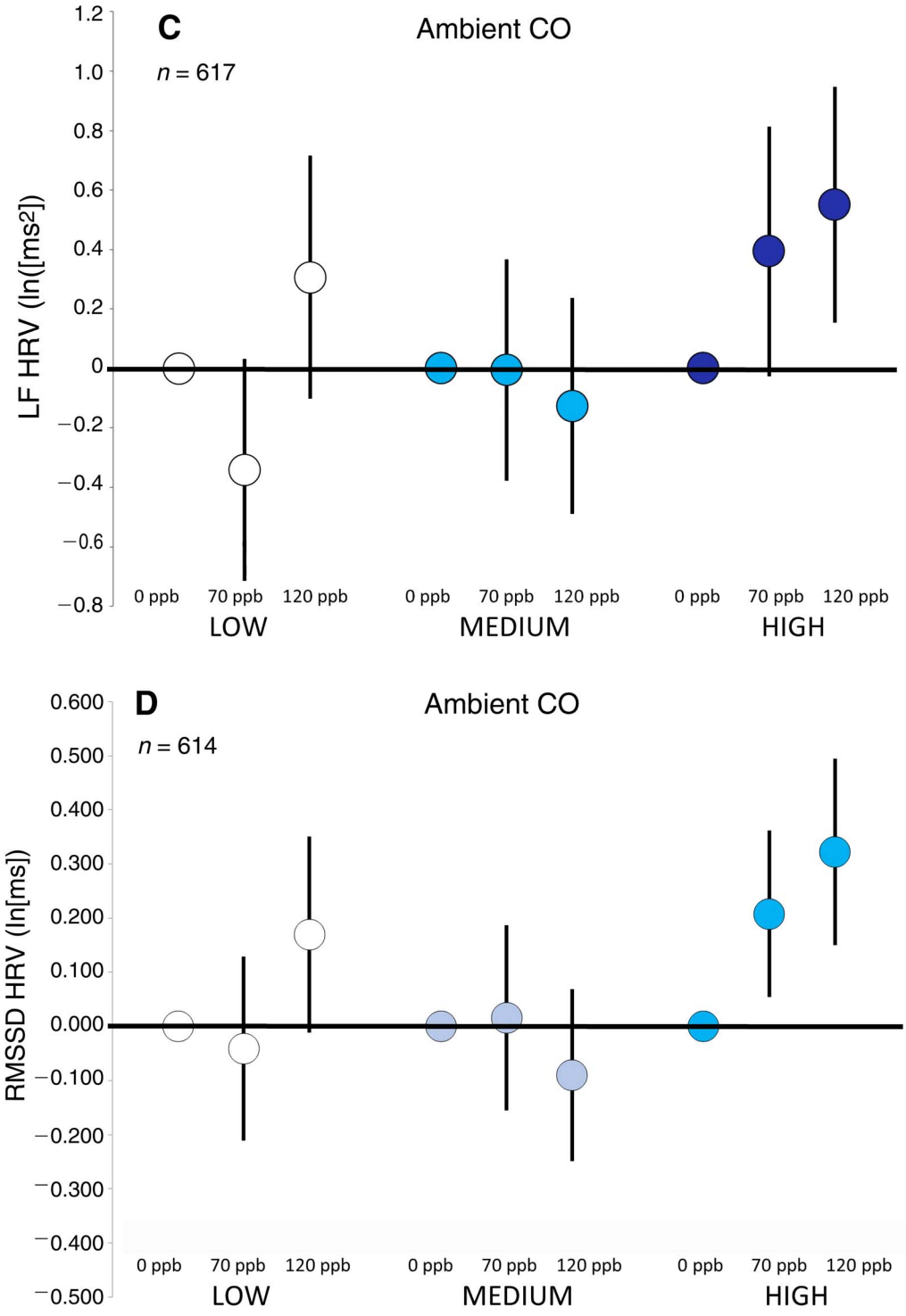


Figure 2. ***(Continued)***

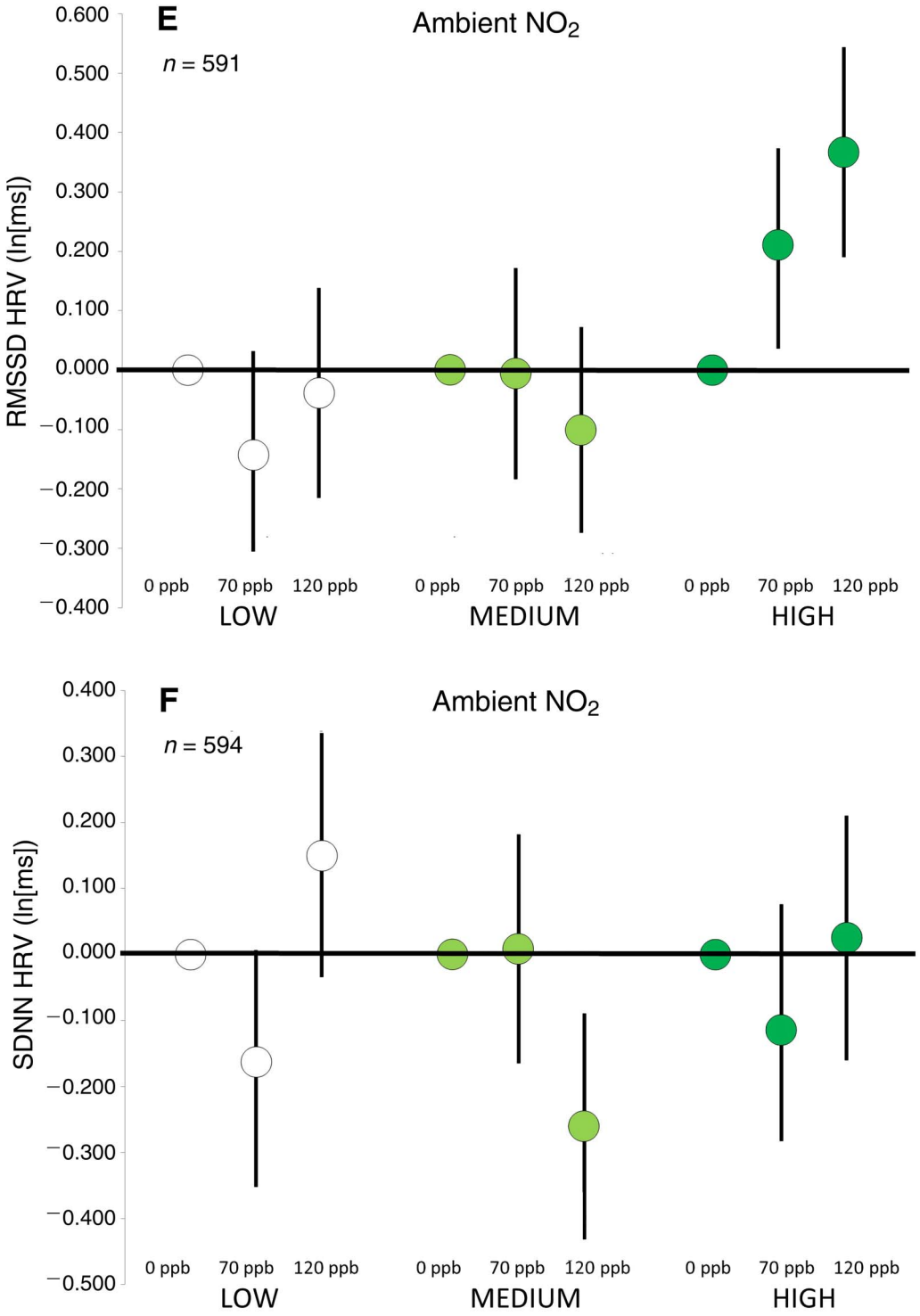


Figure 2. ***(Continued)***

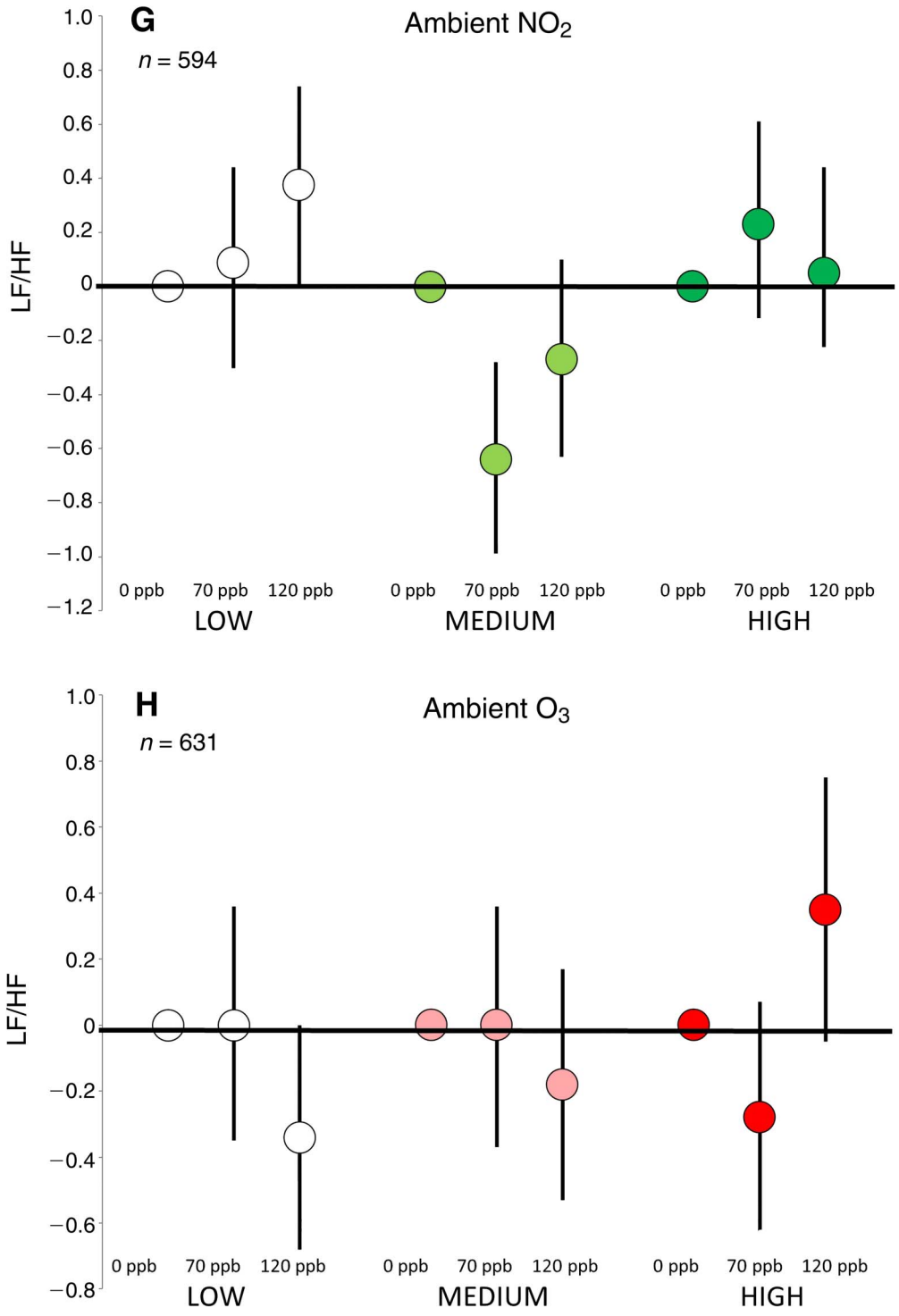


Figure 2. ***(Continued)***

on LF/HF ratio, neither pattern was consistent with Hypothesis #1 or #2 (see all Additional Materials 2 Appendix A).

Summary of Aim 2 Results Because results were not consistent across HRV primary and secondary markers (i.e., different pollutants modifying a controlled O_3/biomarker association and different patterns of biomarker changes associated with controlled O_3 exposures within and across tertiles) there does not appear to be effect modification of the HRV/controlled O_3 exposure by these pollutants.

Repolarization

Ambient SO_2 concentrations in the 72 hours before the pre-exposure visit significantly ($P < 0.001$) modified the pre- to post-exposure change in T-wave amplitude (5 min) associated with the MOSES controlled O_3 exposure (Additional Materials 1, Table 3). However, this pattern was not consistent with either Hypothesis #1 or Hypothesis #2. For example, in the High tertile, there was no exposure–response function, as there was a larger increase in T-wave amplitude (5 min) following the 70-ppb exposure (0.143 ln[μV]; 95% CI, 0.87 to 1.99) than for the 120-ppb exposure (0.014 ln[μV]; 95% CI, −0.038 to 0.066). Further, there was no effect of either the 120-ppb or 70-ppb O_3 exposures on T-wave amplitude (5 min) in the Low tertile. In the Medium tertile, T-wave amplitude (5 min) increased after the 120-ppb exposure (0.081 ln[μV]; 95% CI, 0.014 to 0.147), but not the 70-ppb exposure (Figure 3; Additional Materials 1, Table 3). None of the ambient or PES pollutants significantly modified MOSES controlled O_3 exposure effects on T-wave amplitude (24 hour). However, both ambient $PM_{2.5}$ ($P = 0.027$) and PES NO_2 in the previous 72 hours marginally significantly ($P = 0.041$) modified effects of MOSES controlled O_3 exposures on T-wave amplitude (24 hour)(Additional Materials 1, Table 3). However, for both pollutants the pattern of controlled O_3 exposure effects within and across tertiles was inconsistent with Hypothesis #1 and Hypothesis #2 (Additional Materials 1, Table 3).

Summary of Aim 2 Results Therefore, we see no effect modification by PES or ambient air pollution on the pre- to post-exposure change in repolarization markers following the MOSES controlled O_3 exposures.

ST Segment

None of the PES or ambient air pollutants significantly modified the association between the MOSES controlled

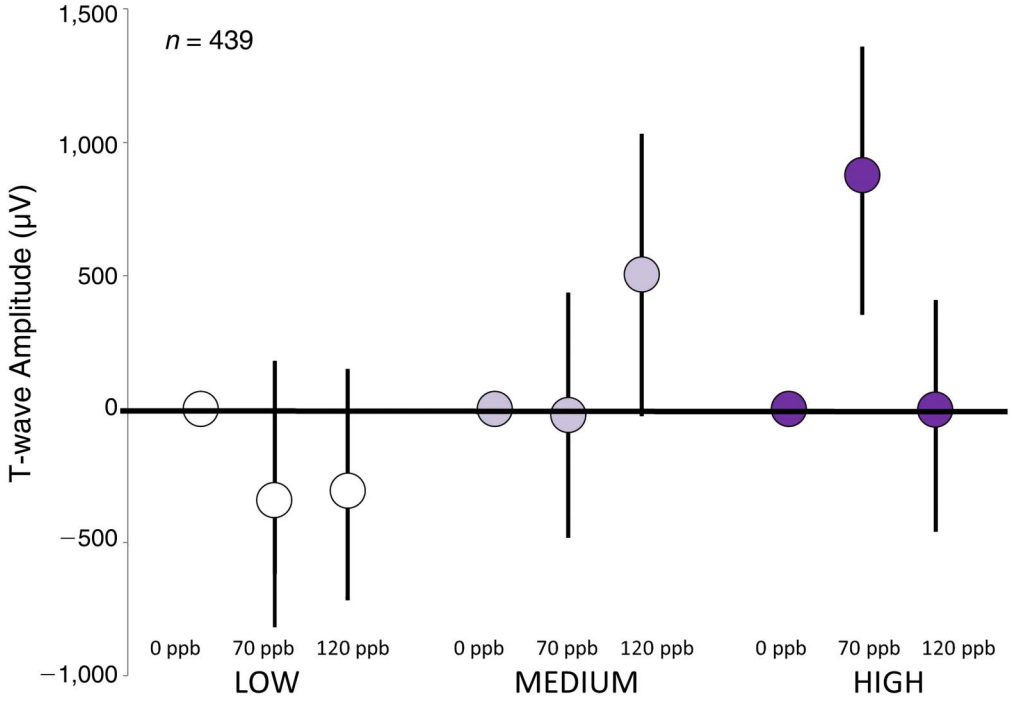


Figure 3. Change in T-wave amplitude (μV) (5-min average) associated with each controlled O_3 exposure, by tertile of ambient pre-exposure SO_2 concentration (lag hours 0–71) (test for interaction $P < 0.001$).

O_3 exposures and pre- to post-exposure changes in either ST in V5 (5 min) or ST in V5 (24 hour) (Additional Materials 1, Table 3). Further, across all pollutants there did not appear to be a pattern of effect modification that was consistent with either Hypothesis #1 or Hypothesis #2.

Summary of Aim 2 Results Therefore, we see no effect modification by PES or ambient air pollution on the pre- to post-exposure change in ST segment markers following the MOSES controlled O_3 exposures.

Vascular Function

Similarly, none of the PES or ambient air pollutants significantly modified effects of MOSES controlled O_3 exposures on pre- to post-exposure changes in either systolic blood pressure (SBP) or flow-mediated dilatation (FMD) (Additional Materials 1, Table 3). Further, across all pollutants there did not appear to be a clear pattern of effect modification that was consistent with either Hypothesis #1 or Hypothesis #2 for either SBP or FMD.

Summary of Aim 2 Results We saw no effect modification of the pre- to post-exposure change in markers of vascular function following the MOSES controlled O_3 exposures, by PES or ambient air pollutants, in the 96 hours before the pre-exposure visit

Systemic Inflammation

None of the PES or ambient air pollutants significantly modified the association between the MOSES controlled O_3 exposures and pre- to post-exposure changes in C-reactive protein (CRP) (Figure 4; Additional Materials 1, Table 3). However, ambient $PM_{2.5}$ concentrations in the 3 hours before the pre-exposure visit did marginally significantly modify this association ($P = 0.012$), with an exposure–response function in the Low tertile. In that tertile, the 120-ppb O_3 exposure caused a 0.219 ln(ms^2) increase in CRP (95% CI, 0.021 to 0.417), whereas the 70-ppb exposure caused a 0.053 ln(ms^2) increase (95% CI, −0.133 to 0.238). However, in the High tertile, the 70-ppb O_3 exposure caused a −0.245 ln(ms^2) decrease in CRP (95% CI, −0.430 to −0.060) compared with the 0-ppb exposure, whereas the 120-ppb exposure caused only a −0.120 ln(ms^2) decrease (95% CI, −0.309 to 0.068).

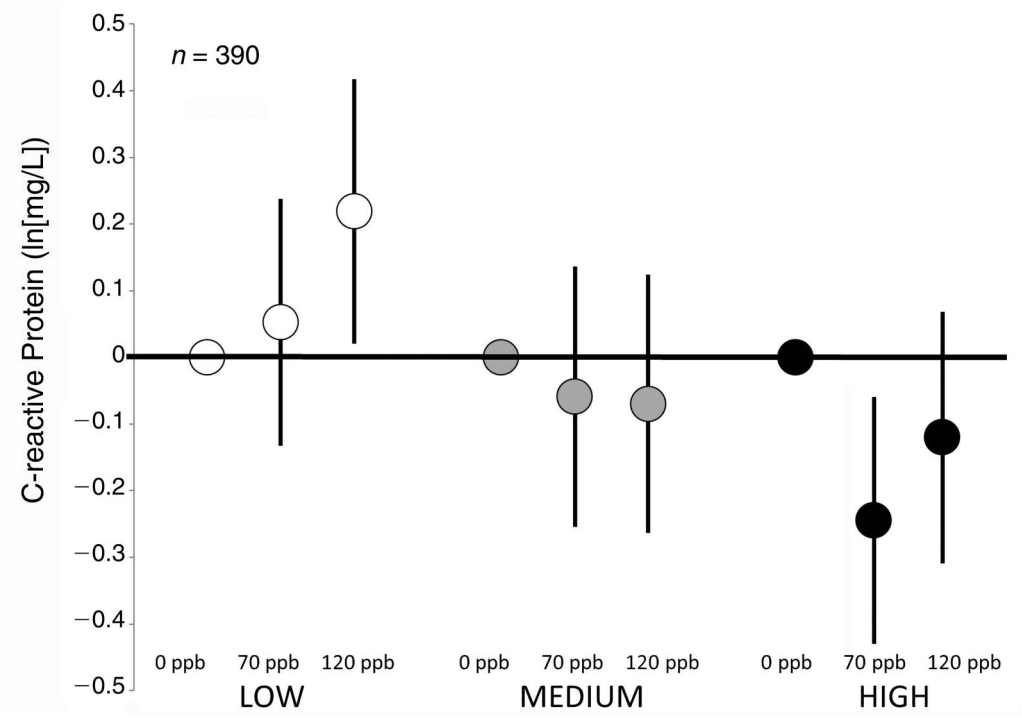


Figure 4. Change in C-reactive protein (ln[mg/L]) associated with each controlled O_3 exposure, by tertile of ambient pre-exposure $PM_{2.5}$ concentration (lag hours 0–2) (test for interaction $P = 0.012$).

Summary of Aim 2 Results Because this pattern was not consistent with either Hypothesis #1 or Hypothesis #2, there was no effect modification of MOSES controlled O_3 exposure effects on systemic inflammation, by ambient or PES air pollutants in the 96 hours before the pre-exposure visit.

Systemic Oxidative Stress

None of the PES or ambient air pollutants significantly modified the association between the MOSES controlled O_3 exposures and pre- to post-exposure changes in nitrotyrosine (Additional Materials 1, Table 3). Further, across all pollutants there did not appear to be a clear pattern of effect modification that was consistent with either Hypothesis #1 or Hypothesis #2.

Summary of Aim 2 Results Therefore, there was no effect modification of MOSES controlled O_3 exposure effects on systemic oxidative stress by ambient or PES air pollutants in the 96 hours before the pre-exposure visit.

Prothrombotic Vascular State

None of the PES or ambient air pollutants significantly modified the association between the MOSES controlled O_3 exposures and pre- to post-exposure changes in microparticle tissue factor activity (Additional Materials 1, Table 3). Further, across all pollutants there did not appear to be a clear pattern of effect modification that was consistent with either Hypothesis #1 or Hypothesis #2. However, although not statistically significant, increased PES O_3 concentrations in the 72 hours before the pre-exposure visit did marginally significantly ($P = 0.012$) modify the change in monocyte–platelet conjugate count following the MOSES controlled O_3 exposures (Figure 5A; Additional Materials 1, Table 3). However, in the Low tertile, the monocyte–platelet conjugate count increased following the 70-ppb O_3 exposure (0.328 ln[count]; 95% CI, 0.078 to 0.578), with a smaller increase following the 120-ppb exposure (0.138 ln[count]; 95% CI, −0.083 to 0.359). Further, in the Medium and High tertiles, there were larger decreases in monocyte–platelet conjugate count following the 70-ppb exposures than the 120-ppb exposures. Thus, this was not consistent with either Hypothesis #1 or Hypothesis #2.

Ambient $PM_{2.5}$ in the previous 96 hours also marginally significantly ($P = 0.035$) modified the change in pre- to post-exposure change in monocyte–platelet conjugate count following the MOSES controlled O_3 exposures, which appeared to be consistent with Hypothesis #2 (Figure 5B; Additional Materials 1, Table 3). In the High tertile, the 120-ppb and 70-ppb O_3 exposures caused decreases of −0.310 ln(count) (95% CI, −0.559 to 0.061) and −0.328 ln(count) (95% CI, −0.585 to −0.071), respectively. There were smaller non-significant increases in monocyte–platelet conjugate count following both the 120-ppb and 70-ppb O_3 exposures in the Low and Medium tertiles.

Summary of Aim 2 Results There was no agreement between the two primary prothrombotic markers, and only one of seven pollutants had an effect modification pattern consistent with either Hypothesis #1 or Hypothesis #2 for monocyte–platelet conjugate count. Therefore, there does not appear to be clear effect modification of the change in prothrombotic markers following the MOSES controlled O_3 exposures by PES or ambient air pollutant concentrations in the 96 hours before the pre-exposure visit.

Pulmonary Function

Increased ambient CO concentrations (3 hours before the pre-exposure visit) marginally significantly ($P = 0.010$) modified the pre- to post-exposure change in FEV_1 following the MOSES controlled O_3 exposures (Figure 6A; Additional Materials 1, Table 3), consistent with Hypothesis #2. In the High tertile, there was a larger decrease in FEV_1 following the 120-ppb exposure (−0.075 L; 95% CI, −0.114 to −0.036) than for the 70-ppb exposure (−0.018 L; 95% CI, −0.055 to 0.019). In the Medium tertile, following the 120-ppb and 70-ppb O_3 exposures, FEV_1 decreased by 0.057 L (95% CI, −0.091 to −0.023) and by 0.054 L (95% CI, −0.090 to −0.018), respectively. In the Low tertile, there was little change in FEV_1 following either the 120-ppb or 70-ppb O_3 exposure. Although only marginally significant ($P = 0.037$), PES NO_2 (72 hours before the pre-exposure visit) followed a similar effect modification pattern. Although not statistically significant, ambient NO_2 concentrations in the 72 hours before the pre-exposure visit also appeared to follow the same pattern (Additional Materials 1, Table 3).

Findings for FVC were similar to FEV_1 and consistent with Hypothesis #2. Increased ambient NO_2 concentrations in the 72 hours before the pre-exposure visit marginally significantly ($P = 0.025$) modified the pre- to post-exposure change in FVC associated with the MOSES controlled O_3 exposures (Figure 6B; Additional Materials 1, Table 3). In the High tertile, FVC decreased following the 120-ppb O_3 exposure (−0.106 L; 95% CI, −0.154 to −0.058), with a smaller reduction following the 70-ppb exposure (−0.047 L; 95% CI, −0.096 to 0.002). In the Medium and Low tertiles, however, FVC did not change following either O_3 exposure. Similarly, PES NO_2 (in 72 hours before the pre-exposure visit) marginally significantly ($P = 0.040$) modified the pre- to post-exposure change in FVC associated with the MOSES controlled O_3 exposures (Additional Materials 1,

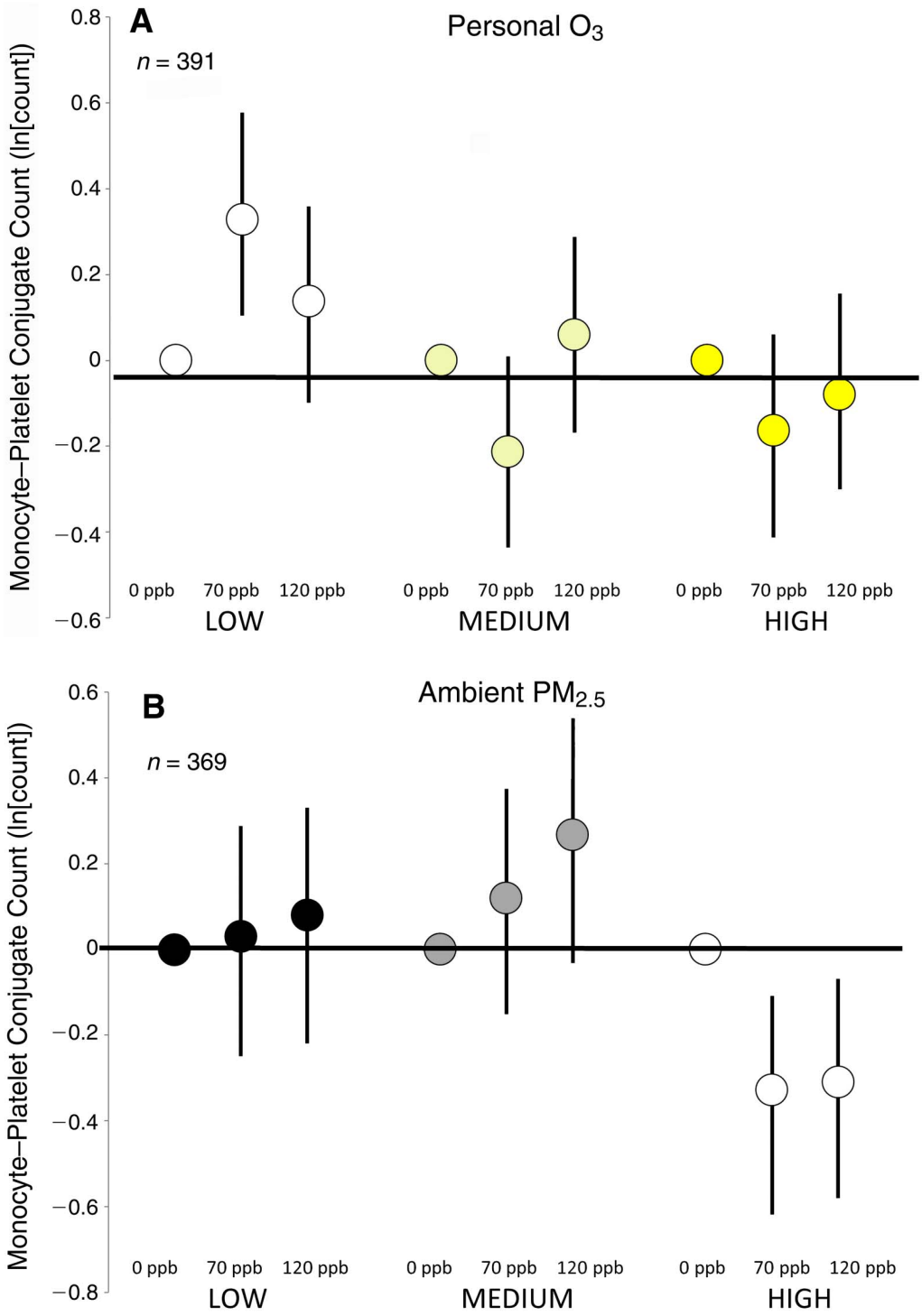


Figure 5. Change in monocyte platelet conjugate counts associated with each controlled O_3 exposure, by tertile of personal or ambient pre-exposure pollutant concentration: (A) ln(count) of monocyte–platelet conjugate for personal O_3 (test for interaction $P = 0.012$) and **(B)** ln(count) of monocyte–platelet conjugate for ambient $PM_{2.5}$ (lag hours 0–95) (test for interaction $P = 0.035$).

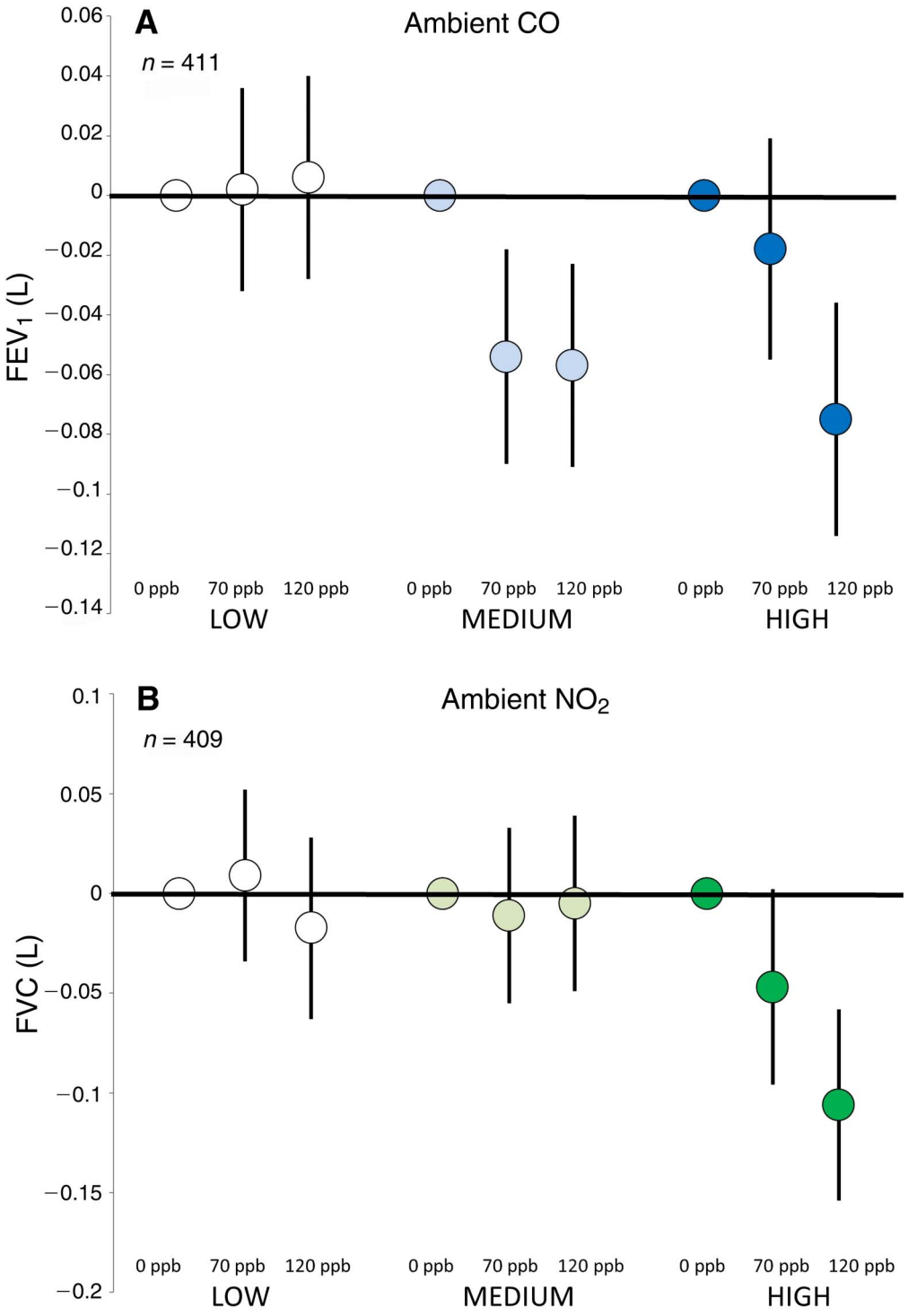


Figure 6. Change lung function measures associated with each controlled O_3 exposure, by tertile of pre-exposure concentration: (A) FEV_1 for ambient CO (lag hours 0–2) (test for interaction $P = 0.010$) and **(B)** FVC for ambient NO_2 (lag hours 0–71) (test for interaction $P = 0.025$).

Table 3), which was also consistent with Hypothesis #2. Although not statistically significant ($P = 0.226$), ambient CO concentrations in the 24 hours before the pre-exposure visit also followed a similar effect modification pattern (Additional Materials 1, Table 3).

Summary of Aim 2 Results The effect of the 120-ppb and 70-ppb MOSES controlled O_3 exposures on markers of pulmonary function (FEV_1 and FVC) appeared to be modified by concentrations of ambient NO_2, CO, and PES NO_2 in the 72 hours before the pre-exposure visit, but not by PES O_3, or ambient O_3, $PM_{2.5}$, or SO_2. Reductions in FEV_1 and FVC, in exposure–response patterns, were generally observed in the High tertile (and Medium tertile in some cases) with no change in these markers in the Low tertile (i.e., consistent with Hypothesis #2). Further, the same pollutants showed the same effect modification patterns for both FEV_1 and FVC.

Lung Injury

None of the PES or ambient air pollutants significantly modified the association between the MOSES controlled O_3 exposures and pre- to post-exposure changes in CC16 (Additional Materials 1, Table 3). Further, across all pollutants there did not appear to be patterns of effect modification that were consistent with either Hypothesis #1 or Hypothesis #2.

Summary of Aim 2 Results Therefore, there was no effect modification of MOSES controlled O_3 exposure effects on lung injury (CC16) by ambient or PES air pollutants in the 96 hours before the pre-exposure visit.

Airway Inflammation

None of the PES or ambient air pollutants significantly modified the association between the MOSES controlled O_3 exposures and pre- to post-exposure changes in sputum PMN % (Additional Materials 1, Table 3). Further, across all pollutants there did not appear to be patterns of effect modification that were consistent with either Hypothesis #1 or Hypothesis #2.

Summary of Aim 2 Results Therefore, there was no effect modification of MOSES controlled O_3 exposure effects on sputum markers of airway inflammation by ambient or PES air pollutants in the 96 hours before the pre-exposure visit.

AIM 3 AND AIM 4

We initially planned to interpret Aim 3 and Aim 4 independently. For example, for Aim 3, we expected to find some adverse changes (i.e., increases or decreases) in pre-exposure biomarker levels associated with increased ambient and personal pollutant concentrations. For Aim 4, we expected to see that increased ambient and personal pollutant concentrations in the 96 hours before the pre-exposure visit would be associated with adverse effects on pre- to post-exposure biomarker changes (e.g., increased pollutant concentrations associated with a decrease in HF from pre- to post-exposure, independent of the controlled O_3 exposure). However, our Aim 3 and Aim 4 results for some of the outcomes suggested an unexpected interplay between these two Aims (i.e., effects of pollutants on pre-exposure biomarkers in Aim 3 may have affected the pollutant-associated pre- to post-exposure changes in Aim 4). For some outcomes, as hypothesized, there was evidence in Aim 3 that increased ambient pollutant concentrations in the 96 hours before the pre-exposure visit were associated with adverse changes in pre-exposure biomarker levels. However for Aim 4, associations were observed in the opposite direction of that hypothesized (i.e., increased ambient pollutant concentrations were associated with an improvement in the biomarker from pre- to post-exposure, independent of the controlled O_3 exposure), as if a "recovery" were occurring during the experimental exposure sessions. Therefore, we present both the Aim 3 and Aim 4 results together, grouped by outcome measure. First, we present results for primary outcomes for an outcome group and, if needed to substantiate any findings for those primary markers, we present findings for secondary outcomes in that same outcome group. The results and the conclusions for Aim 3 and Aim 4, across each outcome group, are also summarized in Table 7.

Heart Rate Variability

Aim 3 Consistent with our a priori hypothesis, IQR increases in ambient O_3 concentrations in the 1, 3, 12, 24, 48, 72, and 96 hours before the pre-exposure visit were all associated with decreased HF (Figure 7A; Additional Materials 1, Table 4). The largest HF decrease was associated with increased O_3 concentrations averaged over the 96 hours before the pre-exposure visit (−0.460 ln[ms^2]; 95% CI, −0.743 to −0.177) for each 10.35-ppb increase in O_3 ($P = 0.002$). In contrast, IQR increases in ambient CO, $PM_{2.5}$, and NO_2 concentrations over these same lags were associated with smaller, nonsignificant increases in HF. However, IQR increases in PES O_3, PES NO_2, and ambient SO_2 concentrations (at the same lags) were not associated with clear changes in HF.

LF showed similar patterns of association with ambient and personal pollutant concentrations (Figure 7B; Additional Materials 1, Table 4). The decreases in LF associated

Table 7. Summary of Findings and Conclusions Across Aims 3 and 4 for Each Outcome Group

Outcome Group	Aim 3 (Pre-exposure biomarker) Associations	Aim 4 (Pre- to post-exposure biomarker change) Associations	Combined Conclusions
Heart rate variability	*Primary* Decreased HF — ambient O_3 Increased HF — ambient CO, $PM_{2.5}$, NO_2 Decreased LF — ambient O_3 Increased LF — ambient CO, $PM_{2.5}$ *Secondary* Decreased RMSSD — ambient O_3 Increased RMSSD — ambient CO, $PM_{2.5}$, NO_2 Decreased SDNN — ambient O_3 Increased SDNN — ambient CO, $PM_{2.5}$, NO_2	*Primary* Increased HF — ambient O_3 Decreased HF — ambient $PM_{2.5}$ Decreased LF — ambient $PM_{2.5}$ *Secondary* Increased RMSSD — ambient O_3 Decreased RMSSD — ambient $PM_{2.5}$ Increased SDNN — ambient O_3 Increased LF/HF — PES NO_2 Decreased LF/HF — ambient O_3	1. Ambient O_3 had effects on pre-exposure HRV. 2. O_3 associations with HF were independent of $PM_{2.5}$, CO, and NO_2. 3. Pre- to post-exposure increases in HRV associated with O_3 may reflect a "recovery" during the exposure sessions.
Repolarization and ST segment	*Primary* No associations with any biomarker	*Primary* No associations with T-wave amplitude Decreased ST segment in V5 — ambient O_3 *Secondary* No associations with ST segment in lead II No associations with ST segment in V2	1. No pollutant associations with repolarization or ST segment.
Vascular function	*Primary* Decreased SBP — PES NO_2 No associations with FMD *Secondary* No associations with DBP Increased vWF — ambient $PM_{2.5}$ Decreased ET-1 — ambient O_3, SO_2 Increased VTI — ambient CO	*Primary* Decreased SBP — PES O_3, $PM_{2.5}$ Increased SBP — PES NO_2 Decreased FMD — ambient O_3 *Secondary* Decreased DBP — ambient $PM_{2.5}$, SO_2 Increased DBP — ambient CO Increased vWF — ambient NO_2, CO, SO_2 Increased ET-1 — ambient CO, SO_2 Increased VTI — PES O_3, ambient O_3 No associations with BAD	1. Directions of biomarker change not consistent with the a priori hypotheses, 2. Pollutants involved not consistent across outcomes. 3. No definitive conclusions about pollutant effects on vascular function.

Table continues next page

Table 7 (*Continued*). Summary of Findings and Conclusions Across Aims 3 and 4 for Each Outcome Group

Outcome Group	AIM 3 (Pre-exposure biomarker) Associations	AIM 4 (Pre- to post-exposure biomarker change) Associations	Combined Conclusions
Systemic inflammation	*Primary* Increased CRP — ambient CO, $PM_{2.5}$, NO_2 *Secondary* Increased fibrinogen — PES NO_2 Decreased P-selectin — ambient O_3 Increased IL-6 — ambient NO_2	*Primary* Decreased CRP — PES NO_2, ambient $PM_{2.5}$, CO *Secondary* Decreased fibrinogen — PES NO_2 Increased fibrinogen — ambient CO Increased P-selectin — ambient O_3, ambient NO_2 Increased IL-6 — ambient $PM_{2.5}$	1. Pre-exposure CRP association with $PM_{2.5}$, CO, and NO_2. 2. Decrease in pre- to post-exposure CRP associated with pollutants suggests "recovery" while the subjects are in the hotel the night before or in the clinical research facility during the exposure session. 3. Secondary markers not supportive of this hypothesis. 4. Findings may reflect random or spurious findings, or they may involve other pathways.
Systemic oxidative stress	*Primary* Decreased nitrotyrosine — ambient NO_2	*Primary* Increased nitrotyrosine — ambient NO_2, CO Decreased nitrotyrosine — ambient O_3	1. No association with pre-exposure or pre- to post-exposure changes in nitrotyrosine. 2. These findings do not support effects of ambient or PES O_3 on nitrotyrosine.
Prothrombotic vascular state	*Primary* Decreased microparticle tissue factor activity — ambient $PM_{2.5}$ Decreased monocyte platelet conjugate count — PES O_3	*Primary* Decreased microparticle tissue factor activity — PES O_3, ambient $PM_{2.5}$ Increased monocyte platelet conjugate count — PES O_3 Decreased monocyte platelet conjugate count — ambient $PM_{2.5}$, SO_2	1. No association between prothrombotic biomarkers and ambient and PES pollutants.
Pulmonary function	*Primary* Decreased FEV_1 — ambient $PM_{2.5}$, CO, NO_2 Decreased FVC — ambient $PM_{2.5}$, CO, NO_2	*Primary* Increased FEV_1 — PES O_3, ambient $PM_{2.5}$, CO Increased FVC — ambient $PM_{2.5}$	1. $PM_{2.5}$, CO, and NO_2 associated with decreased pre-exposure pulmonary function. 2. $PM_{2.5}$ associated with increased pre- to post-exposure FEV_1 and FVC, suggesting a "recovery" during the time in the exposure chamber and laboratory.
Lung Injury	*Primary* No associations with PMN	*Primary* No associations with PMN	1. No pollutant associations with marker of lung injury.

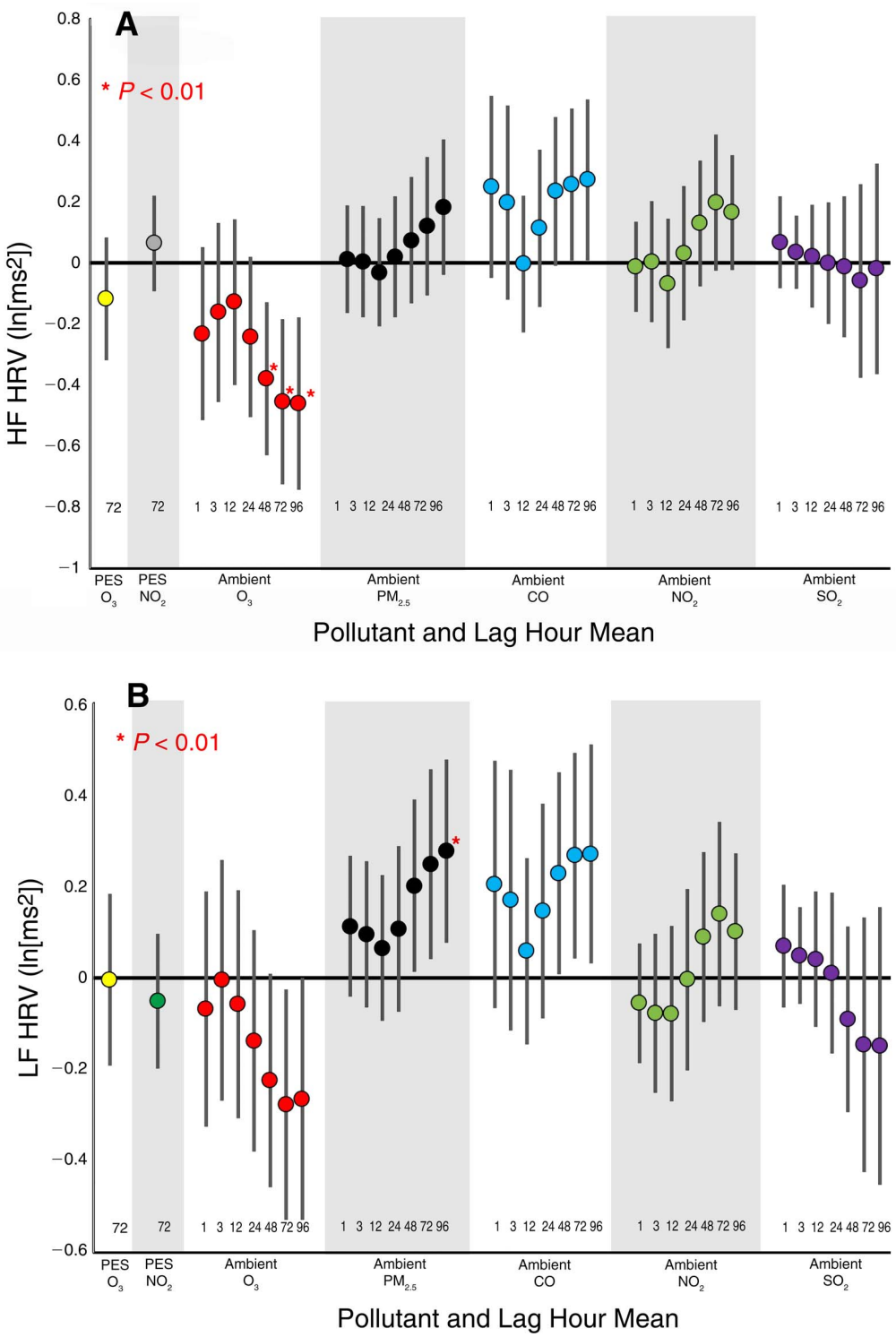
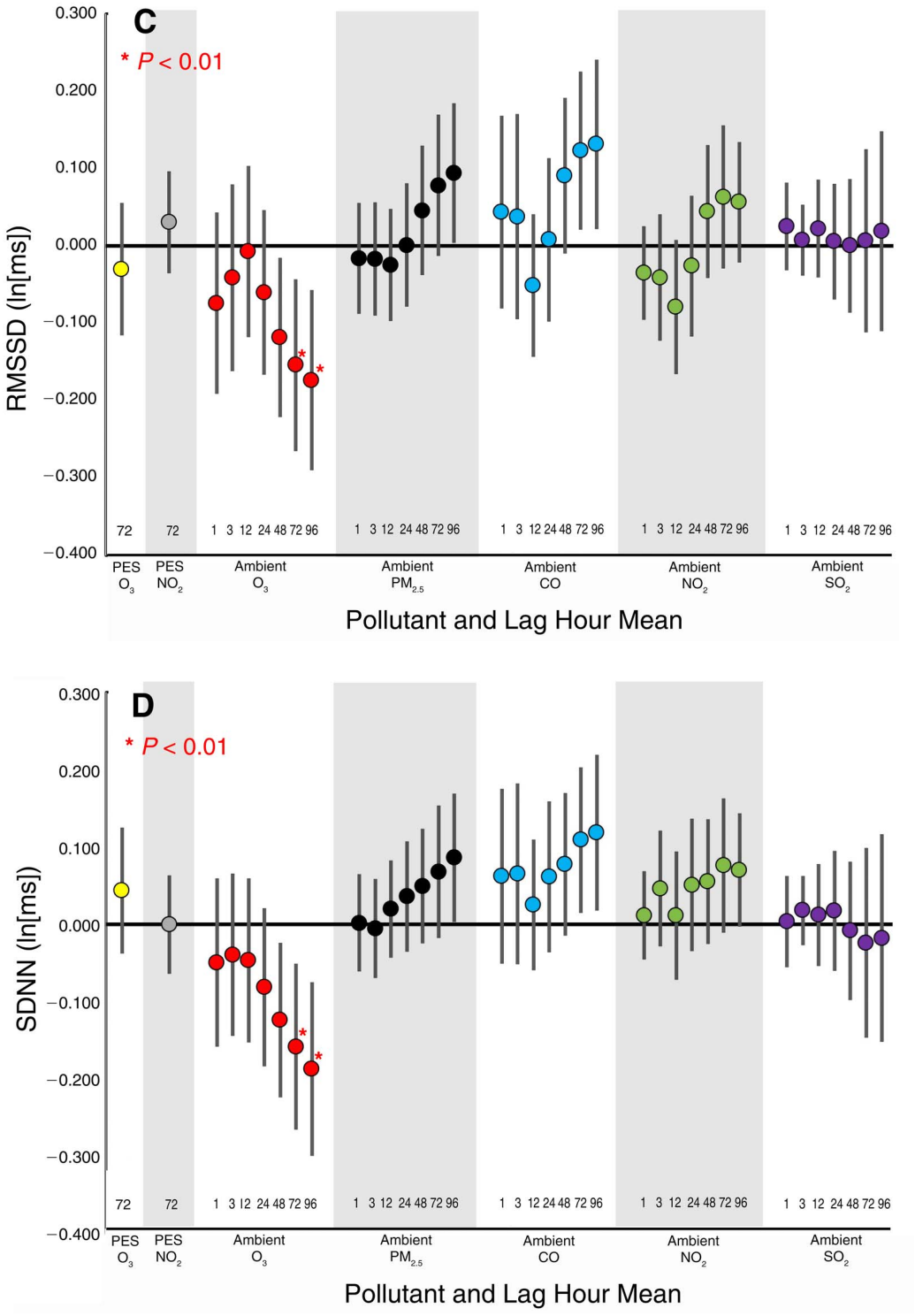


Figure 7. Change in pre-exposure heart rate variability associated with each IQR increase in personal or ambient pre-exposure pollutant concentration, by pollutant and lag hour mean: (A) ln(ms^2) of HF ($P < 0.01$); **(B)** ln(ms^2) of LF ($P < 0.01$); **(C)** ln(ms) of RMSSD ($P < 0.01$); **(D)** ln(ms) of SDNN ($P < 0.01$); and **(E)** LF/HF ratio. (*Figure continues next page.*)



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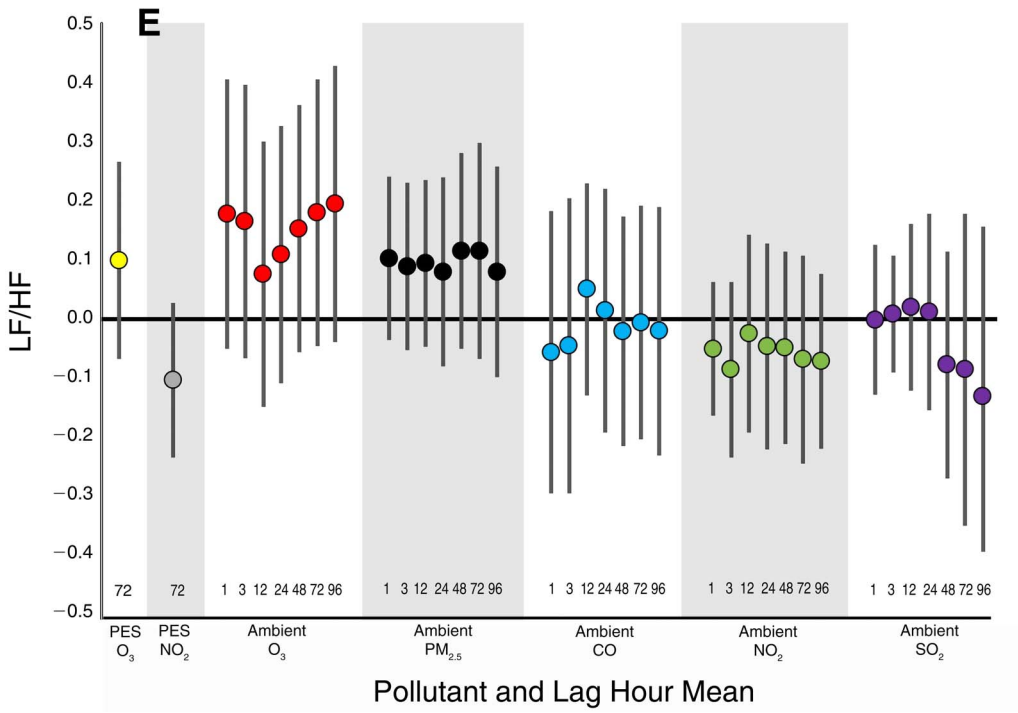

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with IQR increases in O_3 concentrations were smaller in magnitude than the decreases in HF, and were only marginally significant for O_3 averaged over the 96 hours before the pre-exposure visit (−0.265 ln[ms^2]; 95% CI, −0.529 to −0.001 for each 10.35-ppb increase in O_3; $P = 0.049$). The significant increase in LF associated with each IQR increase in ambient $PM_{2.5}$ concentration in the same 96 hours (0.277 ln[ms^2]; 95% CI, 0.077 to 0.477; $P = 0.007$) was similar in magnitude to, but in the opposite direction of, the LF decreases associated with increased O_3 concentrations. Similar to $PM_{2.5}$, increased CO concentrations were associated with increased LF, with the largest LF change associated with increased CO in the 96 hours before the pre-exposure visit. However, there were no such patterns of association with ambient NO_2, ambient SO_2, PES O_3, or PES NO_2.

Because the hypothesized relationships between the primary markers of HRV (HF and LF) and ambient O_3 concentrations were confirmed, we extended the analyses to include the secondary markers of HRV: RMSSD, SDNN, and LF/HF ratio (Figure 7C–E; Additional Materials 2, Appendix B). Decreases in both RMSSD and SDNN were significantly associated with IQR increases in ambient O_3 concentrations, in a pattern similar to that seen for HF and LF. Although not statistically significant, the pattern of RMSSD and SDNN associations with increased ambient $PM_{2.5}$, CO, and NO_2 was again similar to that of HF and LF. There were no such associations with any pollutant and the LF/HF ratio. We therefore concluded that increases in ambient O_3, but not PES O_3 or other pollutants, were associated with decreases in pre-exposure HRV.

Aim 4 Increases in ambient O_3 concentrations were marginally significantly associated with increases in HF across the exposure sessions (Figure 8A; Additional Materials 1, Table 5). The largest increases in HF were associated with increased O_3 concentrations in the 48, 72, and 96 hours before the pre-exposure visit, but none reached statistical significance ($P < 0.01$). Although not statistically significant, decreases in HF were associated with increased $PM_{2.5}$ concentrations at these same time lags. However, these changes in HF associated with increased O_3 concentrations were opposite in direction from those seen in Aim 3, where increases in O_3 concentrations were associated with decreases in pre-exposure HF levels.

Increases in $PM_{2.5}$ concentrations across all lag times were associated with decreases in LF, with the largest LF decrease associated with each 4.3-μg/m^3 increase in $PM_{2.5}$ concentration in the 96 hours before the pre-exposure visit

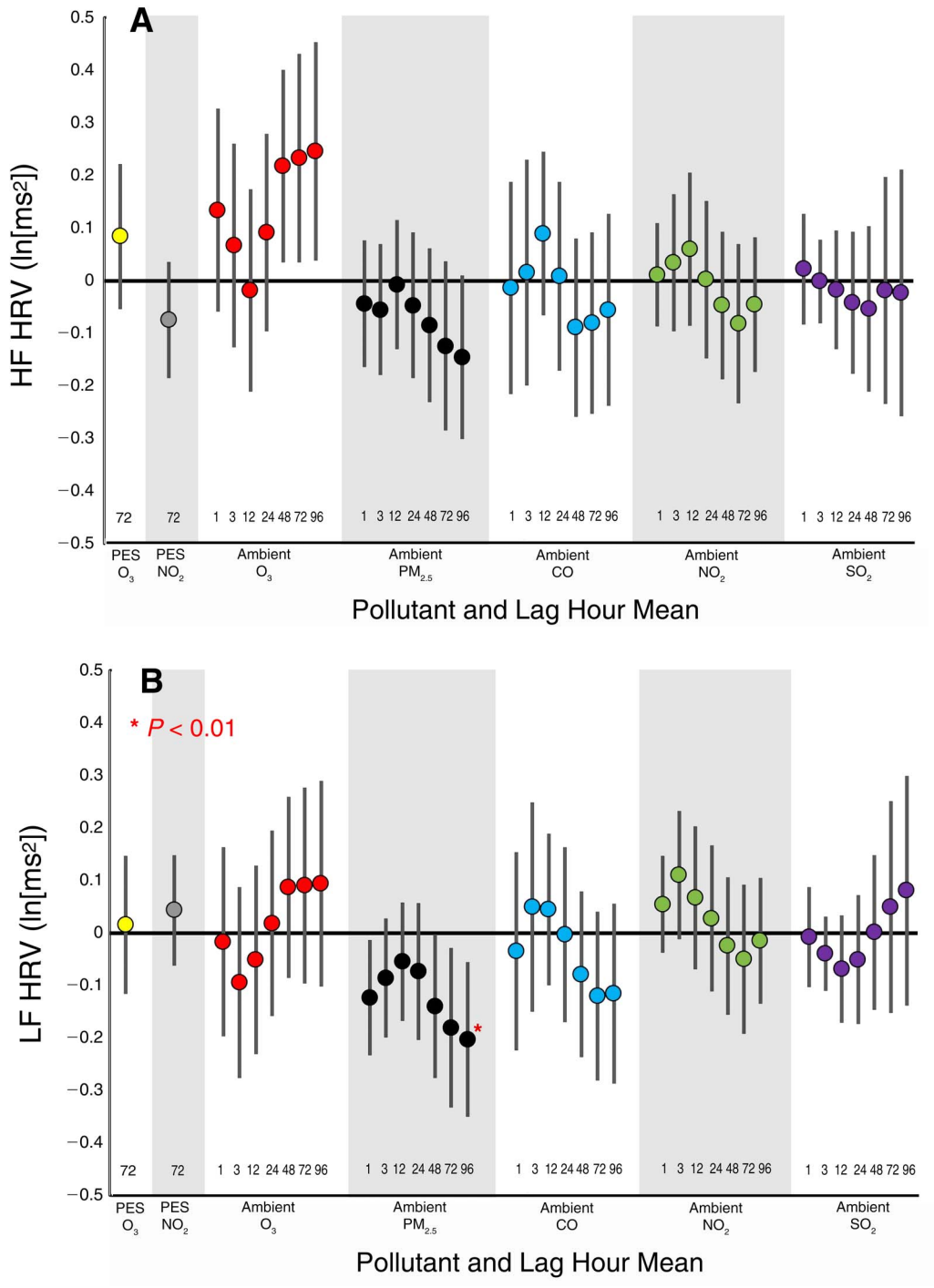
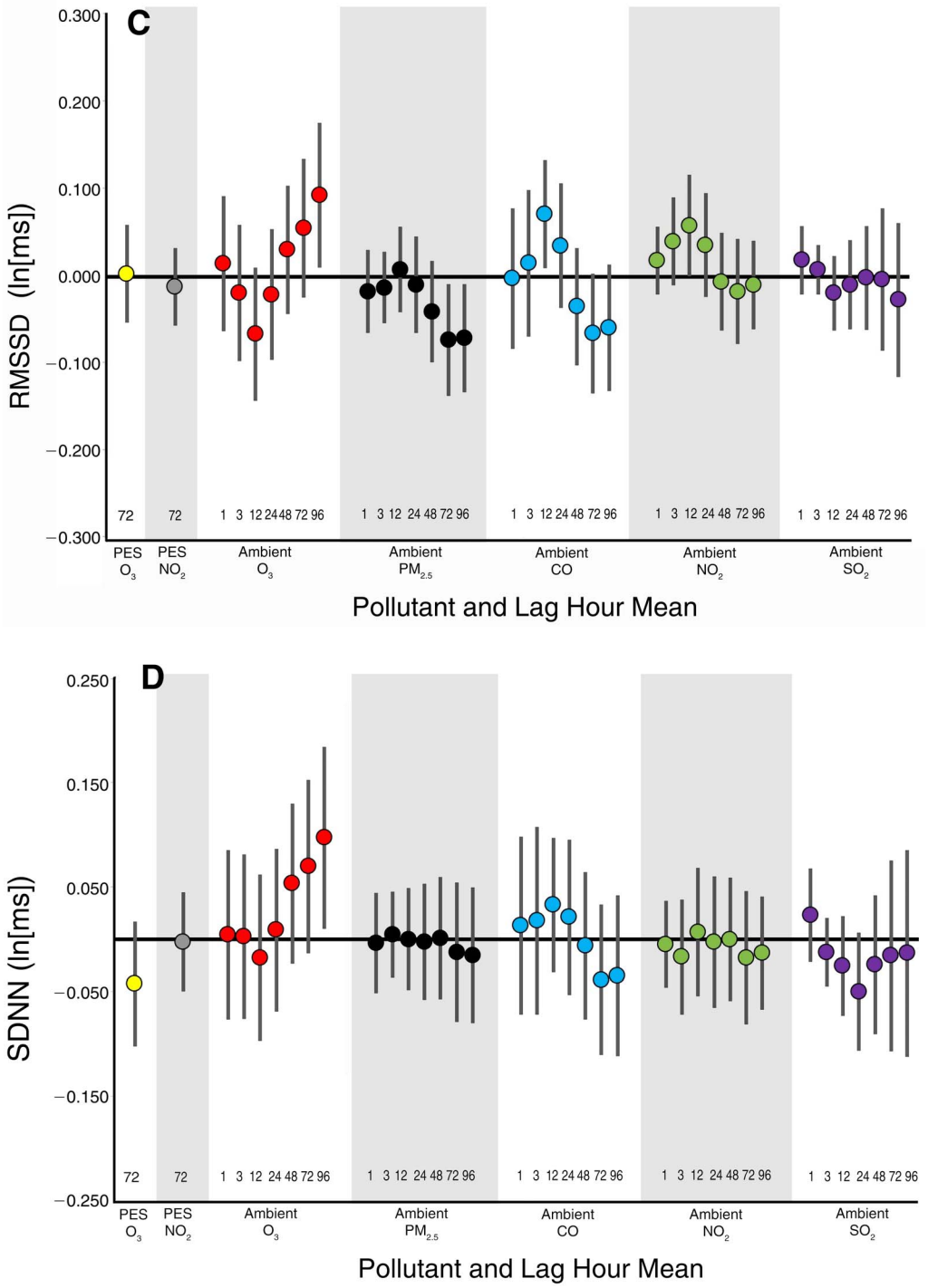


Figure 8. Difference in the pre- to post-exposure change in heart rate variability measures associated with each IQR increase in personal or ambient pre-exposure pollutant concentration, by pollutant and lag hour mean: (A) $\ln(ms^2)$ of HF (5 min); **(B)** $\ln(ms^2)$ of LF (5 min) ($P < 0.01$); **(C)** ln(ms) of RMSSD (5 min); **(D)** ln(ms) of SDNN (5 min); and **(E)** LH/HF ratio (5 min). *(Figure continues next page.)*



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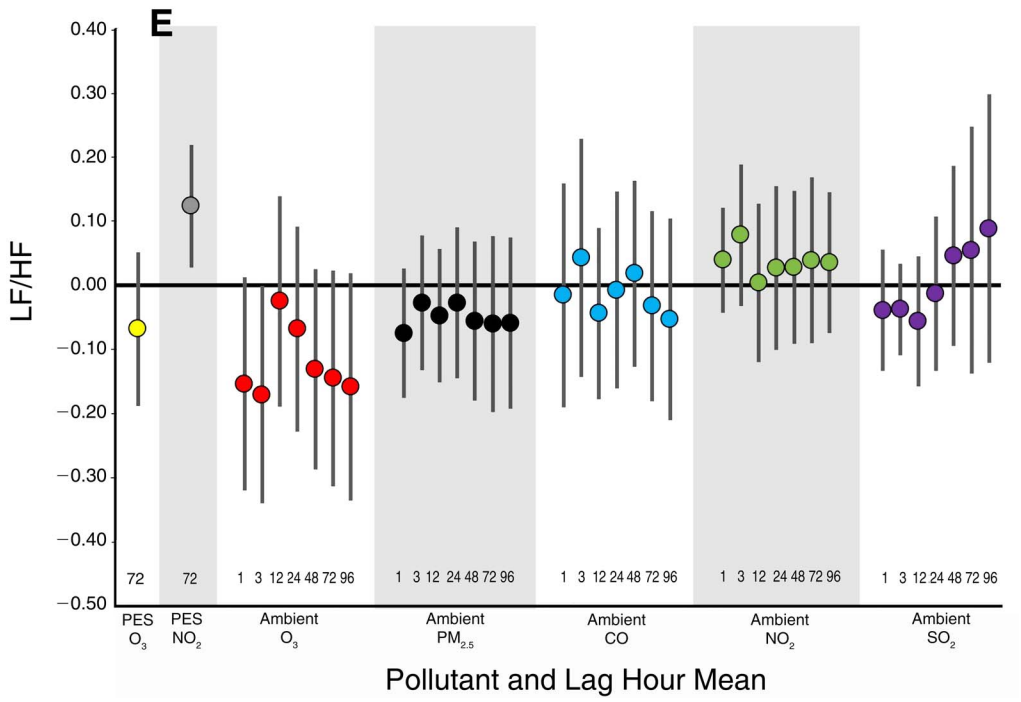

Figure 8. ***(Continued).***

(−0.203 ln[ms^2]; 95% CI, −0.350 to −0.055; P = 0.007) (Figure 8B; Additional Materials 1, Table 5). However, there were no clear patterns of LF associations with the other pollutants.

Because of these findings with the primary HRV outcomes (HF and LF), we analyzed the secondary outcome markers for HRV. Increased RMSSD was marginally significantly associated with increased ambient O_3 concentrations, with the largest RMSSD change associated with each 10.3-ppb increase in O_3 concentration in the 96 hours before the pre-exposure visit (0.093 ln[ms^2]; 95% CI, 0.010 to 0.176; P = 0.027). However, decreased RMSSD levels were marginally significantly associated with increased $PM_{2.5}$ concentrations with the largest RMSSD change associated with each 4.3-μg/m^3 increase in $PM_{2.5}$ concentration in the same 96 hours (−0.071 ln[ms^2]; 95% CI, −0.132 to −0.009; P = 0.026) (Figure 8C; Additional Materials 2, Appendix C). Increased SDNN levels were also marginally significantly associated with increased ambient O_3 concentrations (Figure 8D; Additional Materials 2, Appendix C), but not with other pollutants. Increased LF/HF ratio was marginally significantly associated with increased PES NO_2, but decreased LF/HF ratio was marginally significantly associated with increased ambient O_3 in the 3 hours before the pre-exposure visit (Figure 8E; Additional Materials 2, Appendix C). The patterns and direction of change for RMSSD and SDNN were consistent with the findings for HF. The marginally significant decrease in LF/HF ratio associated with O_3 was consistent with the observed larger increase in HF than in LF.

Because we found increased HF and LF associated with increased O_3 concentrations, but decreased HF and LF associated with increased $PM_{2.5}$, CO, and NO_2 in some single-pollutant models, we ran a series of two-pollutant models for the Aim 3 primary HRV outcomes (HF and LF). We then compared the change in O_3 concentration when adjusting for each of the other pollutants (Additional Materials 2, Appendix D) to that from the single-pollutant model (Additional Materials 1, Table 4). When including $PM_{2.5}$ in the model, the estimated change in HF associated with increased O_3 concentrations at each lag time was little changed and robust. In the two-pollutant model, HF decreased −0.472 ln(ms^2) (95% CI, −0.777 to −0.167, P = 0.003) with each 10.35-ppb increase in O_3 concentration averaged over the 96 hours before the pre-exposure visit, compared with −0.460 ln(ms^2); (95% CI, −0.743, to −0.177, P = 0.002) for the single-pollutant model with O_3 alone. The O_3 associations were also little changed after adjustment for

CO and NO_2 concentrations (Additional Materials 2, Appendix D).

The changes in LF associated with increased O_3 concentrations in the 1 to 96 hours before the pre-exposure visit (when also adjusting for $PM_{2.5}$, CO, or NO_2 concentrations at the same time lag), although smaller than in the single-pollutant models, were still in the same direction and the inference based on them remained the same (Additional Materials 2, Appendix D). However, when adjusting for $PM_{2.5}$, the decrease in LF associated with each IQR increase in ambient O_3 concentration in the 96 hours before the pre-exposure visit was essentially removed (−0.017 ln[ms^2]; 95% CI, −0.290 to 0.255; $P = 0.172$). As reported above, in the single-pollutant model, each IQR increase in ambient O_3 concentration in the 96 hours before the pre-exposure visit was associated with a larger decrease in LF (−0.265 ln[ms^2]; 95% CI, −0.529 to −0.001; $P = 0.049$) (Figure 7B; Additional Materials 1, Table 4). The decreases in LF associated with IQR increases in ambient O_3 concentration in the 48, 72, and 96 hours before the pre-exposure visit were reduced somewhat in the two-pollutant models when adjusting for the CO concentration at the same time lag, but not when adjusting for NO_2 (Additional Materials 2, Appendix D).

Summary of Aim 3 and Aim 4 Findings Thus, these findings suggest that as hypothesized, increased ambient O_3 concentrations had adverse effects on pre-exposure HRV levels, with reductions in HF, LF, RMSSD, and SDNN. The O_3 associations with HF were independent of $PM_{2.5}$, CO, and NO_2 in two-pollutant models. The HRV increases from pre- to post-exposure associated with the same increased O_3 concentrations in Aim 4 may reflect a "recovery" of HRV during the exposure sessions.

Cardiac Repolarization and ST Segment

Aim 3 The primary markers of repolarization were T-wave amplitude and ST segment in V5. We found no clear patterns of association and no significant associations between increased pollutant concentrations and these outcomes at the pre-exposure visit (Additional Materials 1, Table 4; Additional Materials 3, Appendix E, Figure S1).Therefore, secondary markers of repolarization were not examined for this Aim.

Aim 4 There were also no clear patterns of association and no significant or marginally significant associations between increased pollutant concentrations and pre- to post-exposure change in T-wave amplitude (Additional Materials 1, Table 5; Additional Materials 3, Appendix F, Figure S1). Decreases in the ST segment in V5 were associated with increased ambient O_3 concentrations across all lags, with the largest change associated with each 10.3-ppb increase in O_3 concentration in the 96 hours before the pre-exposure visit (−3.0 μV; 95% CI, −5.0 to −1.0; $P = 0.003$) (Additional Materials 1, Table 5; Additional Materials 2, Figure S2). There were no clear patterns of association and no significant associations with the other pollutants (Additional Materials 1, Table 5).

We next examined associations with the secondary ST segment outcomes for Aim 4. Neither changes in ST segment in lead II nor ST segment in V2 were associated with increased ambient pollutant concentrations at any lag, or with increased PES O_3 or PES NO_2 (Additional Materials 2, Appendix C).

Summary of Aim 3 and Aim 4 Findings Based on these findings, we concluded that increased ambient and personal pollutant concentrations were not associated with adverse changes in ECG markers of repolarization or ST segment.

Vascular Function

Aim 3 Each 9.4-ppb increase in PES NO_2 concentration was associated with a significant reduction in SBP (−1.457 mm Hg; 95% CI, −2.490 to −0.425; $P = 0.006$) (Figure 9A; Additional Materials 1, Table 4). However, there were no clear patterns of association or significant SBP changes associated with any ambient pollutant or PES O_3. There were no significant associations between FMD, the other primary marker of vascular function, and any pollutant concentration, although all estimated changes in FMD associated with increased concentrations of ambient O_3, $PM_{2.5}$, and SO_2, at all lag times, were positive (Figure 9B; Additional Materials 1, Table 4).

Aim 4 As seen in Figure 10A, each 4.1-ppb increase in PES O_3 concentration was marginally significantly associated with decreases in SBP across the exposure sessions (−1.1 mm Hg; 95% CI, −2.0 to −0.1; $P = 0.032$) (Additional Materials 1, Table 5). Each 9.4-ppb increase in PES NO_2 concentration was marginally significantly associated with increases in SBP across the exposure session (0.8 mm Hg; 95% CI, 0.0 to 1.6; $P = 0.043$). Increased $PM_{2.5}$ concentrations in the 1, 3, 12, and 24 hours before the pre-exposure visit were associated with decreases in SBP, with the largest SBP change associated with each 5.8-μg/m^3 increase in $PM_{2.5}$ concentration in the 3 hours before the pre-exposure visit (−1.1 mm Hg; 95% CI, −2.0 to −0.3; $P = 0.010$). Increased ambient O_3 concentrations were associated with non-significant decreases in FMD, the other primary marker of vascular function, for most lag

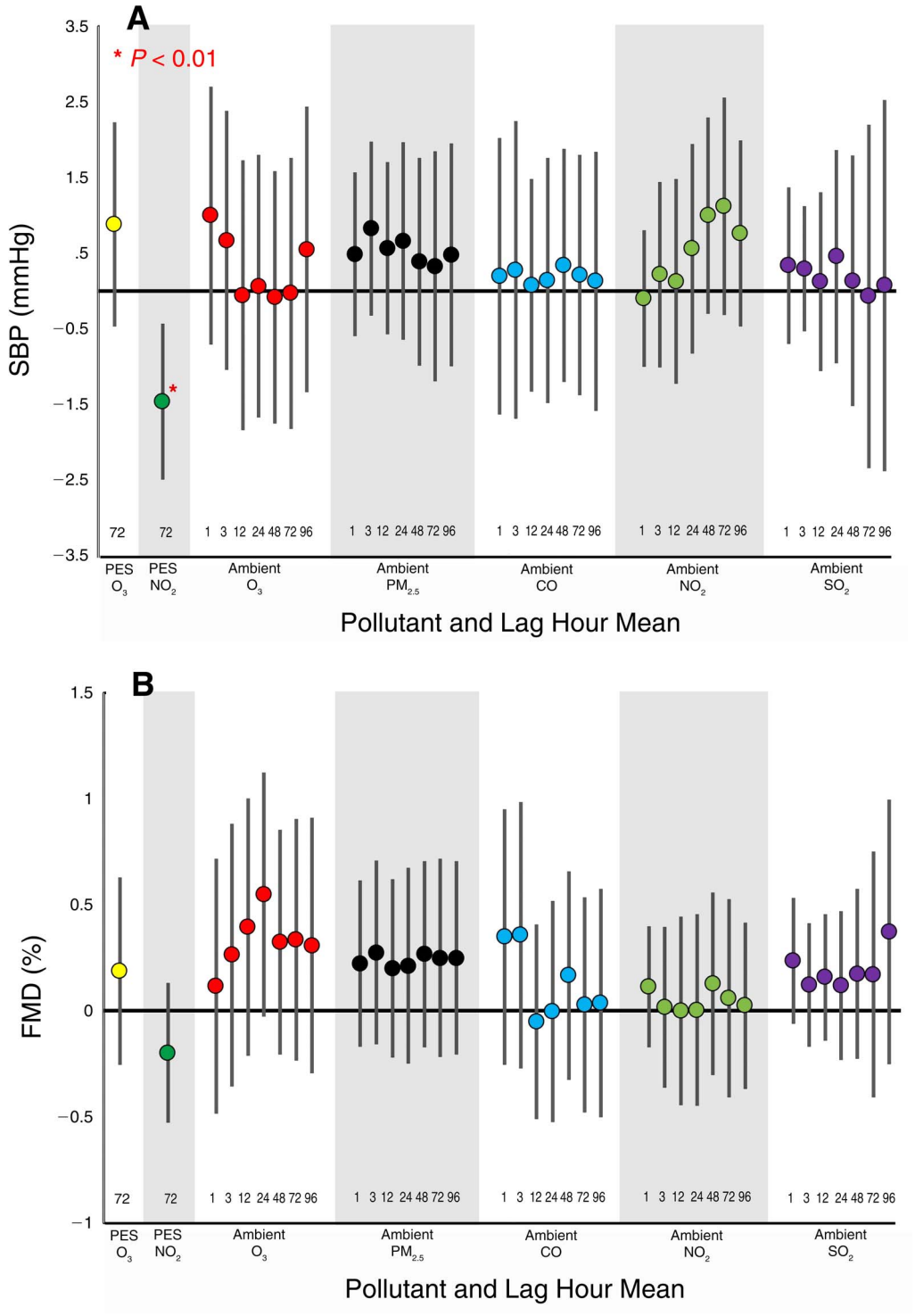

Figure 9. Change in pre-exposure blood pressure and flow associated with each IQR increase in personal or ambient pre-exposure pollutant concentration, by pollutant and lag hour mean: (A) systolic blood pressure ($P < 0.01$) and **(B)** flow-mediated dilatation.

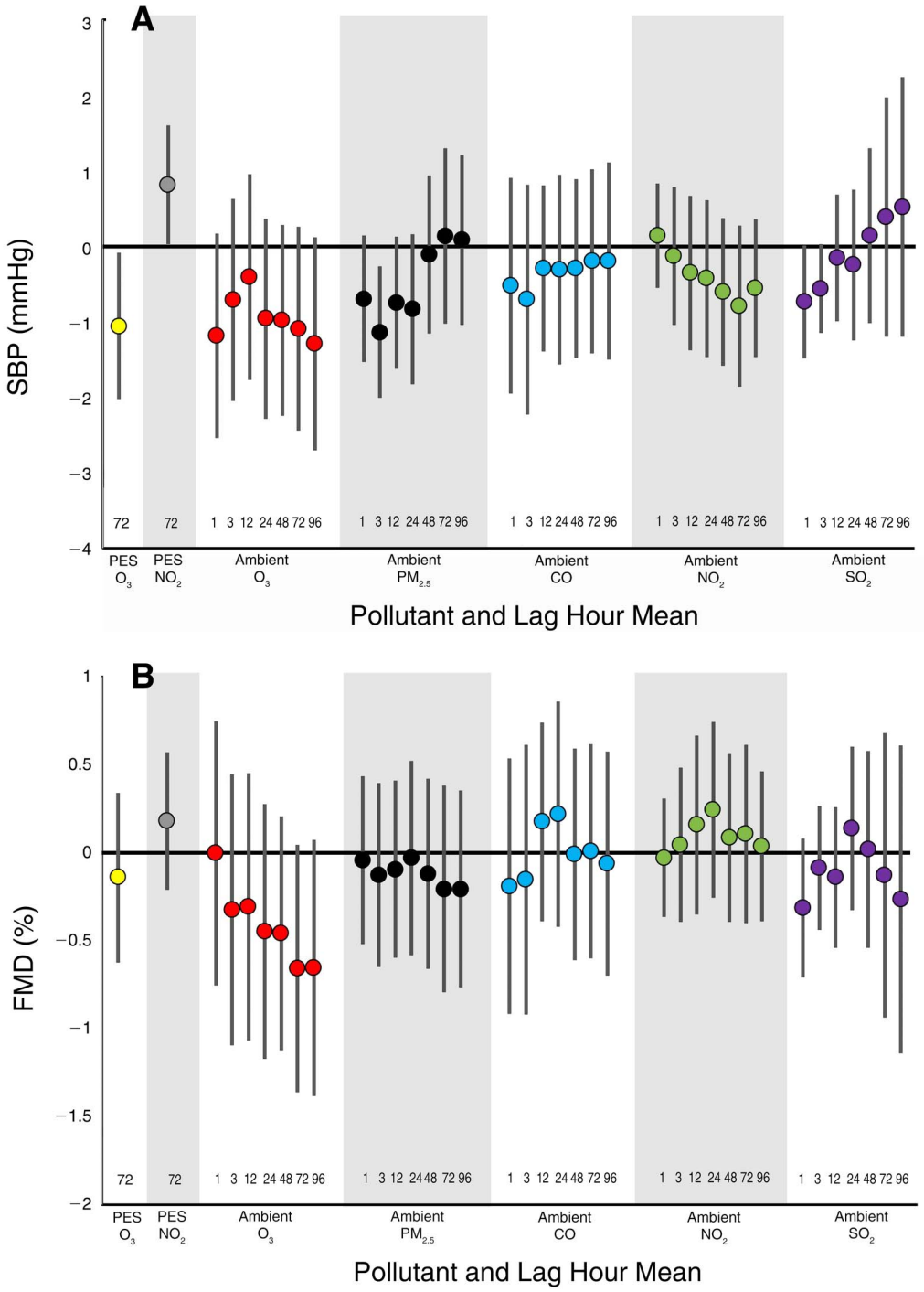

Figure 10. Difference in the pre- to post-exposure change in blood pressure and flow associated with each IQR increase in personal or ambient pre-exposure pollutant concentration, by pollutant and lag hour mean: (A) systolic blood pressure and **(B)** flow-mediated dilatation.

times (Figure 10B; Additional Materials 1, Table 5). However, there were no patterns of association or significant associations between FMD and any other pollutant.

The finding that increases in PES NO_2 were associated with decreased pre-exposure SBP (Aim 3) and increased SBP across the exposure sessions raises the possibility of a vasodilator effect of increases in personal NO_2 exposure, with "recovery" during the exposure sessions. We therefore examined results for the secondary markers of vascular function: diastolic BP (DBP), plasma von Willebrand factor (vWF), endothelin-1 (ET-1). We also examined the secondary outcomes from brachial artery ultrasound testing: velocity–time interval (VTI) and brachial artery diameter (BAD). The findings for these secondary markers are shown in tables in Additional Materials 2 and in figures in Additional Materials 3. There were no associations between increases in any ambient pollutant, PES O_3, or PES NO_2 and pre-exposure DBP in any Aim 3 analysis (Additional Materials 3; Appendix E, Figure S3). Increases in pre-exposure vWF were associated with each IQR increase in $PM_{2.5}$ concentration in the 1 hour before the pre-exposure visit (0.13 ln[ng/mL]; 95% CI, 0.003 to 0.265; P = 0.045) (Additional Materials 3; Appendix E, Figure S4). However, decreased ET-1 was associated with IQR increases in ambient O_3 concentration at all lags, with the largest change associated with increased ambient O_3 in the 3 hours before the pre-exposure visit (−0.10 pg/mL; 95% CI, −0.16 to −0.04; P = 0.001). Decreased ET-1 was marginally significantly associated with increases in SO_2 concentration in the 1 hour before the pre-exposure visit (Additional Materials 3; Appendix E, Figure S5). Increased VTI was marginally significantly associated with increases in CO concentration in the 1 hour before the pre-exposure visit (4.1 cm; 95% CI, 0.15 to 7.97; P = 0.042) (Additional Materials 3; Appendix E, Figure S6). There were no clear patterns of association or statistically significant associations between BAD and any pollutant.

For the Aim 4 secondary markers of vascular function, decreases in DBP across the exposure sessions were associated with increased $PM_{2.5}$ concentrations in the 1, 3, 12, and 24 hours before the pre-exposure visit, with the largest decrease associated with $PM_{2.5}$ in the 3 hours before the pre-exposure visit (−0.8 mm Hg; 95% CI, −1.4 to −0.2; P = 0.008) (Additional Materials 3; Appendix F, Figure S3). Increases in DBP across the exposure session were associated with increased CO concentrations at all lag times, with the largest decrease associated with CO in the 96 hours before the pre-exposure visit (1.2 mm Hg; 95% CI, 0.4 to 2.1; P = 0.006). Decreased DBP across the exposure session was marginally significantly associated with increased SO_2 concentration in the 24 hours before the pre-exposure visit (−0.8 mm Hg; 95% CI, −1.5 to −0.1; P = 0.027), with smaller non-significant changes at other lag hours. Increased vWF concentrations across the exposure session were marginally significantly associated with increases in ambient NO_2 concentration in the 48 and 96 hours before the pre-exposure visit (48-hour lag: 0.12 ln[ng/mL]; 95% CI, 0.02 to 0.22; P = 0.026), with a similar pattern of association with CO and SO_2 (Additional Materials 3, Appendix F, Figure S4).

Increased ET-1 across the exposure session was marginally significantly associated with increases in CO concentration in the 1 hour and 3 hours before the pre-exposure visit, with the largest change at 3 hours (0.07 pg/mL; 95% CI, 0.01 to 0.13; P = 0.033) (Additional Materials 3, Appendix F, Figure S5). Increased ET-1 across the exposure session was also associated with increases in SO_2 concentration at all lag times, with the largest increase associated with SO_2 in the 96 hours before the pre-exposure visit (0.09 pg/mL; 95% CI, 0.02 to 0.16; P = 0.007). Increased VTI across the exposure session was significantly associated with increases in PES O_3 (3.8 cm; 95% CI, 1.1 to 6.5; P = 0.007), with similar sized effects (although not statistically significant) associated with increased ambient O_3 in the 1, 3, 12, and 24 hours before the pre-exposure visit (Additional Materials 3, Appendix F, Figure S6). There were no clear patterns of association or significant associations between pre- to post-exposure changes in BAD and any pollutant at any lag time (Additional Materials 3, Appendix F, Figure S7).

Summary of Aim 3 and Aim 4 Findings Although there were a few associations for some of the primary and secondary markers of vascular function for both Aim 3 and Aim 4, the directions of change were often not consistent with the a priori hypotheses, and the pollutants involved were not consistent across outcomes. This lack of consistency across pollutants and outcomes precludes any definitive conclusions about effects of prior pollutant exposure on vascular function.

Systemic Oxidative Stress

Aim 3 Increased ambient NO_2 concentrations were associated with decreased pre-exposure nitrotyrosine levels at all lag times, with the largest change associated with each 7.9-ppb increase in ambient NO_2 concentration in the 12 hours before the pre-exposure visit (−0.094 ln[nM]; 95% CI, −0.164 to −0.023; P = 0.010) (Figure 11; Additional Materials 1, Table 4). However, the direction of this change was the opposite of that hypothesized. Further, there were no patterns of association between pre-exposure nitrotyrosine and increases in other ambient or PES pollutant concentrations.

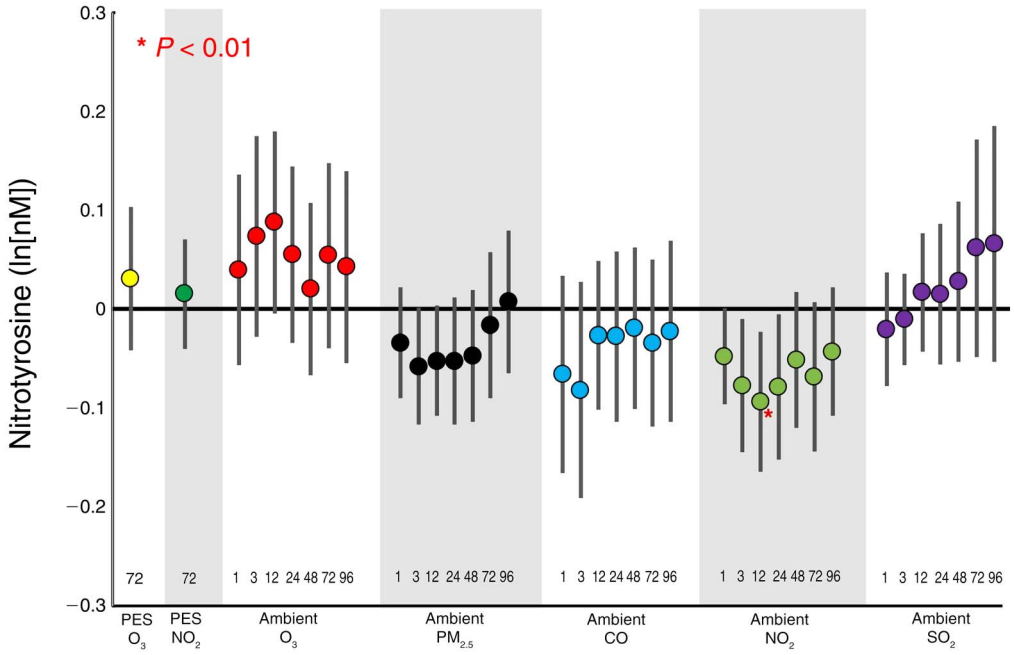

Figure 11. Change in pre-exposure nitrotyrosine (ln[nM]) associated with each IQR increase in personal or ambient pre-exposure pollutant concentration, by pollutant and lag hour mean ($P < 0.01$).

Aim 4 Increased ambient NO_2 concentrations at all lags were associated with increases in nitrotyrosine, with the largest increase in the 12 hours before the pre-exposure visit (0.55 ln[nM]; 95% CI, 0.015 to 0.095; $P = 0.007$) (Figure 12; Additional Materials 1, Table 5). Similarly, increased nitrotyrosine was associated with marginally significantly increased ambient CO concentrations in the 1 hour before the pre-exposure visit (0.070 ln[nM]; 95% CI, 0.12 to 0.129; $P = 0.019$). However, increased ambient O_3 concentrations at all lags were associated with decreases in nitrotyrosine, reaching marginal significance at the 72-hour lag (−0.072 ln[nM]; 95% CI, −0.131 to −0.013; $P = 0.017$). There were no patterns of association or significant nitrotyrosine associations with any other pollutant (Figure 12; Additional Materials1, Table 5).

Summary of Aim 3 and Aim 4 Findings We concluded that there was no clear association between increased pollutant concentrations and either pre-exposure levels or pre- to post-exposure changes in nitrotyrosine. We previously reported in MOSES 1 that nitrotyrosine decreased in response to the experimental O_3 exposures, with marginal statistical significance, and that the direction of effect was opposite of that hypothesized. The current findings with ambient or PES O_3 are not consistent with the changes in MOSES 1. Similar to MOSES 1, these MOSES 2 findings do not support effects of ambient or PES O_3 on nitrotyrosine.

Systemic Inflammation

Aim 3 Increased CO concentrations across all lag times were associated with increased pre-exposure levels of C-reactive protein (CRP; the primary marker of systemic inflammation), with the largest change associated with each 0.14-ppb increase in CO concentration in the 3 hours before the pre-exposure visit (0.206 ln[mg/L]; 95% CI, −0.005 to 0.417; $P = 0.056$) (Figure 13; Additional Materials 1, Table 4). Similarly, increased CRP levels were marginally significantly associated with increases in $PM_{2.5}$ in the 3 hours before the pre-exposure visit and NO_2 in the 12 hours before the pre-exposure visit.

This direction of change is consistent with our a priori hypothesis, so analyses were undertaken of secondary markers of systemic inflammation (fibrinogen, P-selectin, and IL-6; see Additional Materials 2, Appendix A). Increased pre-exposure fibrinogen was significantly associated with each 9.3-ppb increase in PES NO_2 concentration (0.100 ln[ng/mL]; 95% CI, 0.025 to 0.176; $P = 0.009$), but there were no clear patterns of association with other pollutants (Additional Materials 3, Appendix E, Figure S7).

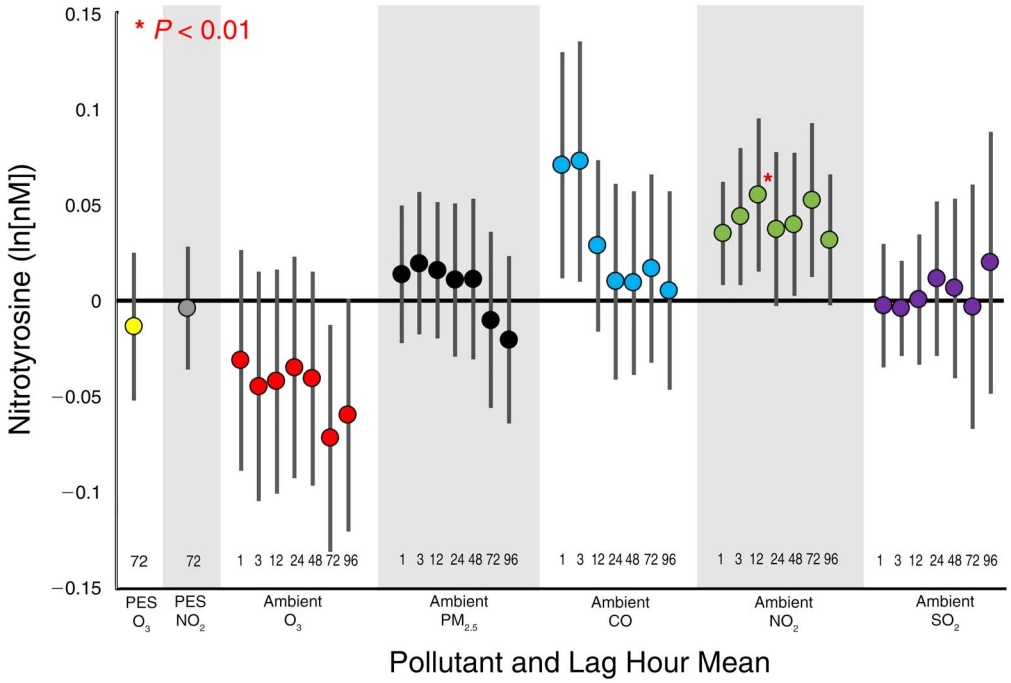

Figure 12. Difference in the pre- to post-exposure change in nitrotyrosine (ln[nM]) associated with each IQR increase in personal or ambient pre-exposure pollutant concentration, by pollutant and lag hour mean ($P < 0.01$).

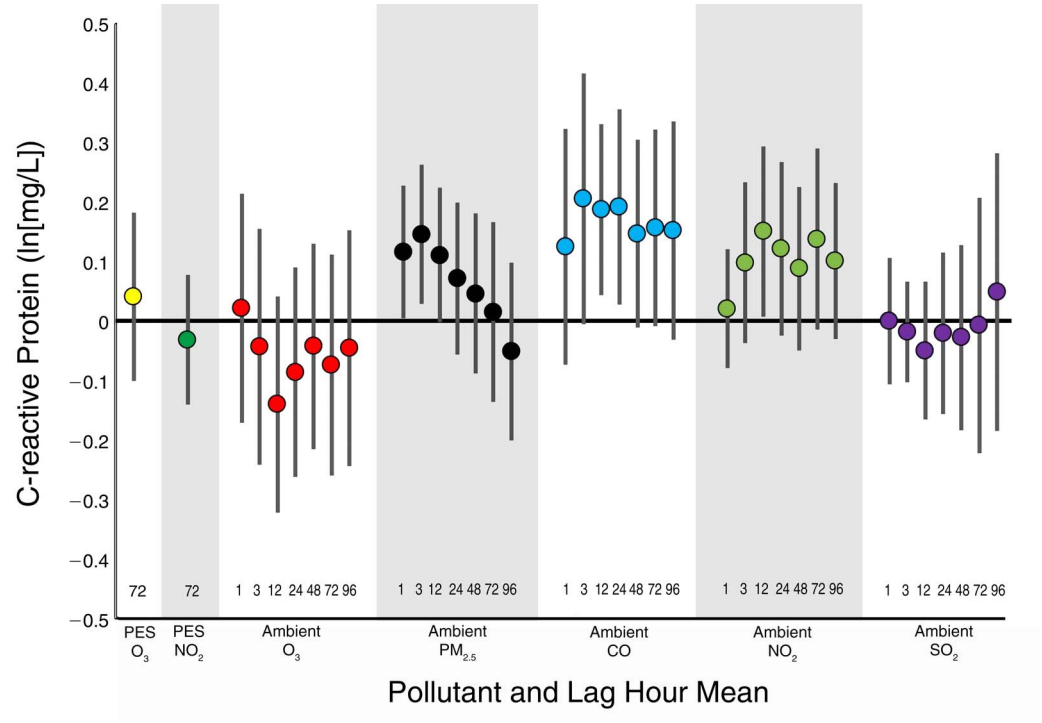

Figure 13. Change in pre-exposure C-reactive protein (ln[mg/L]) associated with each IQR increase in personal or ambient pre-exposure pollutant concentration, by pollutant and lag hour mean.

Decreased P-selectin was significantly associated with each 15.2-ppb increase in ambient O_3 concentration in the 1 hour before the pre-exposure visit (−0.171 ln[ng/mL]; 95% CI, −0.295 to −0.047; $P = 0.007$), but not with other pollutants (Additional Materials 3, Appendix E, Figure S8). Increased IL-6 was marginally significantly associated with each 4.2-ppb increase in NO_2 concentration in the 96 hours before the pre-exposure visit (0.128 ln[pg/mL], 95% CI, 0.011 to 0.246; $P = 0.033$), but there were no clear patterns of association between IL-6 and any pollutant (Additional Materials 3, Appendix E, Figure S9).

Aim 4 As shown in Figure 14A and in Additional Materials 1, Table 5, decreased CRP across the exposure session was significantly associated with each 9.4-ppb increase in PES NO_2 in the 72 hours before the pre-exposure visit (−0.084 ln[mg/L]; 95% CI, −0.135 to −0.034; $P = 0.001$) and each 5.5-ppb increase in ambient NO_2 concentration in the 3 hours before the pre-exposure visit (−0.083; 95% CI, −0.139 to −0.027; $P = 0.004$). Further, each 5.8-μg/m^3 increase in ambient $PM_{2.5}$ concentration in the 3 hours before the pre-exposure visit was also associated with decreased CRP (−0.067 ln[mg/L]; 95% CI, −0.119 to −0.016; $P = 0.011$). Although not significant, there was a similar pattern of association between CRP and increased CO concentrations at the same lags.

Because of these findings, we examined secondary outcomes related to systemic inflammation for Aim 4. Decreased fibrinogen across the exposure session was significantly associated with each 9.4-ppb increase in PES NO_2 concentration in the 72 hours before the pre-exposure visit (−0.091 [μg/mL]; 95% CI, −0.148 to −0.034; $P = 0.002$) (Figure 14B; Additional Materials 2, Appendix C), but increased fibrinogen across the exposure session was significantly associated with increases in CO concentration, with the largest increase in the 96 hours before the pre-exposure visit (0.161 [μg/mL]; 95% CI, 0.069 to 0.252; $P = 0.001$) (Figure 14B). Increased P-selectin across the exposure sessions was significantly associated with each 15.2-ppb increase in ambient O_3 concentration in the 1 hour before the pre-exposure visit (0.162 [ng/mL]; 95% CI, 0.056 to 0.267; $P = 0.003$), and marginally significantly associated with each 7.9-ppb increase in ambient NO_2 concentration in the 12 hours before the pre-exposure visit (0.083 ln[ng/mL]; 95% CI, 0.007 to 0.158; $P = 0.032$) (Figure 14C; Additional Materials 2, Appendix C). Increased IL-6 across the exposure sessions was marginally significantly associated with each 5.4-μg/m^3 increase in $PM_{2.5}$ concentration in the 12 hours before the pre-exposure visit (0.337 pg/mL; 95% CI, 0.036 to 0.638; $P = 0.028$) , and decreased IL-6 across the exposure sessions was associated with each 0.108-ppm increase in ambient CO concentration in the 96 hours before the pre-exposure visit (−0.450 pg/mL; 95% CI, −0.886 to −0.015; $P = 0.043$) (Figure 14D; Additional Materials 2, Appendix C).

Summary of Aim 3 and Aim 4 Findings The Aim 3 findings with CRP suggest the possibility of a pro-inflammatory pollutant effect with $PM_{2.5}$, CO, and NO_2. The Aim 4 findings with CRP — a decrease across the exposure sessions — support such a pro-inflammatory effect with "recovery" while the subjects are indoors (either in the hotel the night before or in the clinical research facility during the exposure session) and breathing cleaner air. However, the results with the secondary inflammatory markers are not consistently supportive of this hypothesis, with some pollutants associated with decreases and others with increases in the secondary markers of inflammation. These findings may reflect, in part, random or spurious findings or there may be more complicated processes involving multiple pathways that cannot be deciphered with these data.

Prothrombotic Vascular State

Aim 3 Decreased microparticle tissue factor activity, a primary marker of a prothrombotic vascular state, was associated with increased ambient $PM_{2.5}$ concentrations across all lag times, with the largest decrease associated with each 4.6-μg/m^3 increase in $PM_{2.5}$ concentration in the 48 hours before the pre-exposure visit (−0.034 pg/mL; 95% CI, −0.063 to −0.005; $P = 0.024$) (Figure 15A; Additional Materials 1, Table 4). Decreases in monocyte–platelet conjugate count, the other primary prothrombotic marker, were marginally significantly associated with each 4.1-ppb increase in PES O_3 concentration (−0.107; 95% CI, −0.213 to −0.002; $P = 0.046$). However, there were no clear patterns of association with any pollutant (Figure 15B, Additional Materials 1, Table 4).

Aim 4 There were no significant associations between pollutant concentrations and the pre- to post-exposure change in either of the primary prothrombotic outcomes. However, decreased microparticle tissue factor activity across the exposure session was marginally significantly associated with each 4.1-ppb increase in PES O_3 concentration in the 72 hours before the pre-exposure visit (−0.024 pg/mL; 95% CI, −0.047 to −0.001; $P = 0.041$) while increased microparticle tissue factor activity across the exposure session was associated with each 4.6-μg/m^3 increase in $PM_{2.5}$ concentration in the 48 hours before the pre-exposure visit (0.026 pg/mL; 95% CI, 0.002 to 0.050; $P = 0.037$) (Figure 16A; Additional Materials 1, Table 5). Increased monocyte–platelet conjugate count across the

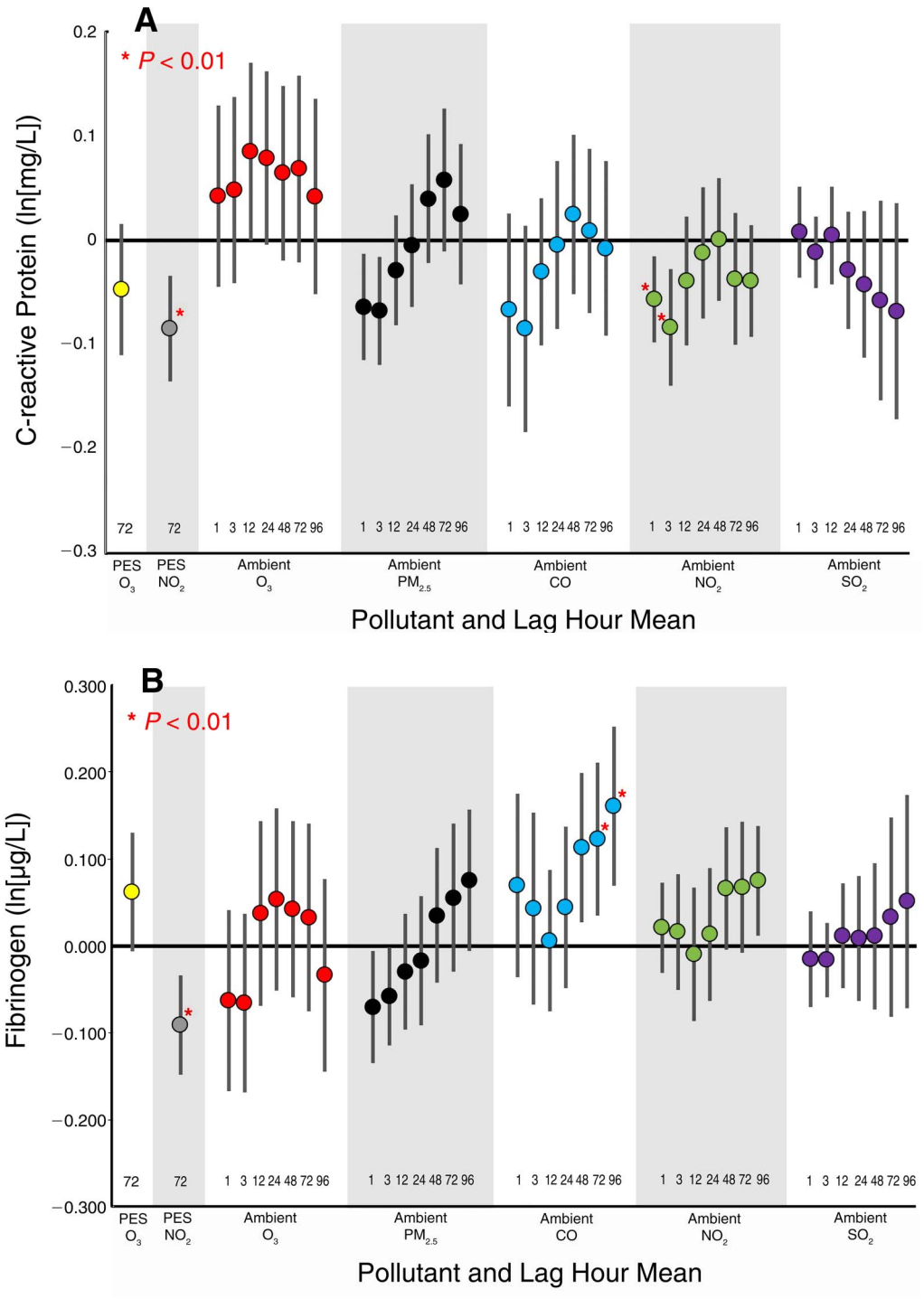

Figure 14. Difference in the pre- to post-exposure change in inflammatory markers associated with each IQR increase in personal or ambient pre-exposure pollutant concentration, by pollutant and lag hour mean: (A) C-reactive protein (ln[mg/L]) ($P < 0.01$); **(B)** fibrinogen (ln[µg/L]) ($P < 0.01$); **(C)** P-selectin (ln[ng/mL]) ($P < 0.01$); and **(D)** IL-6 (ln[pg/mL]). *(Figure continues next page.)*

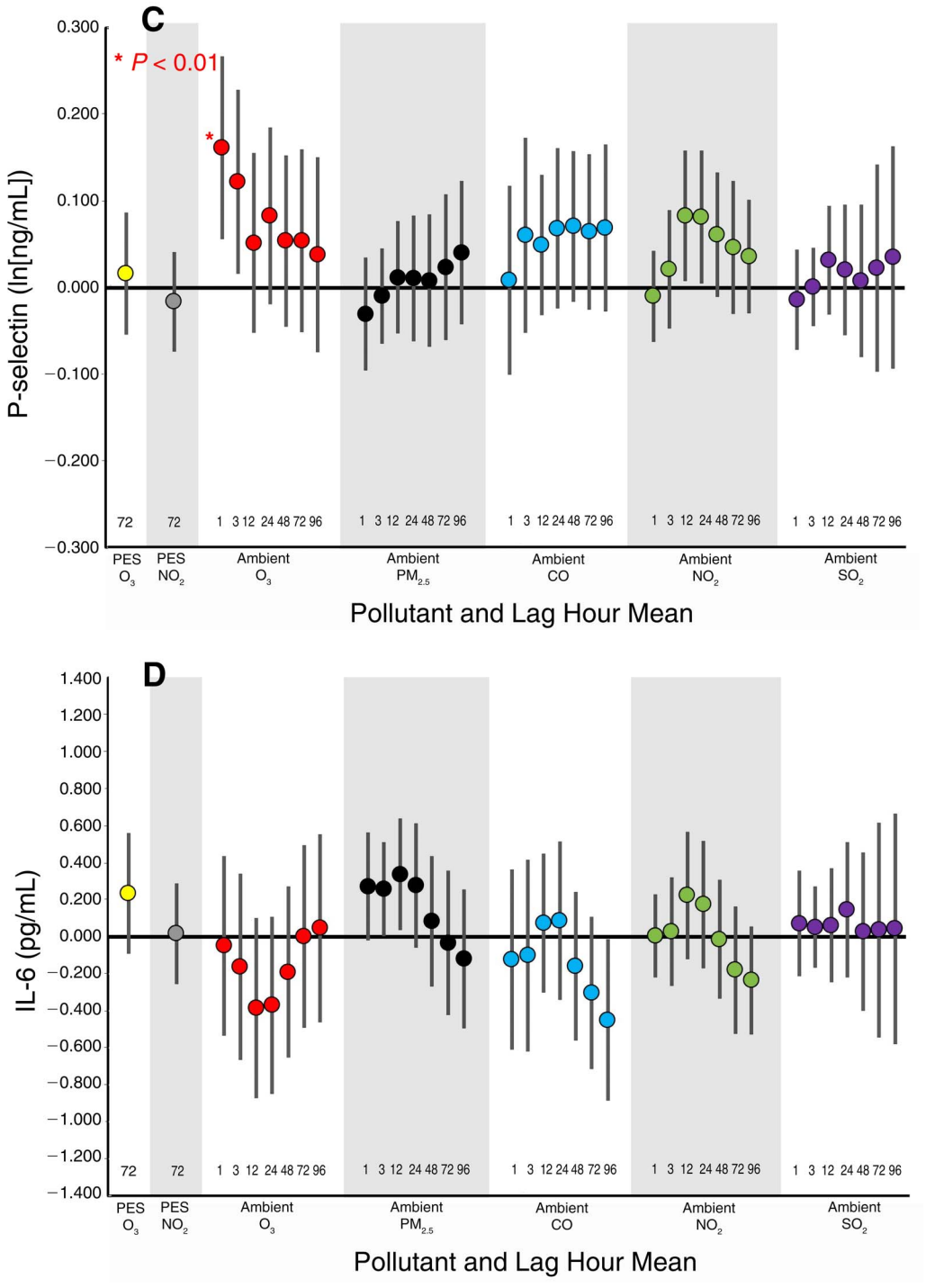

Figure 14 ***(Continued).***

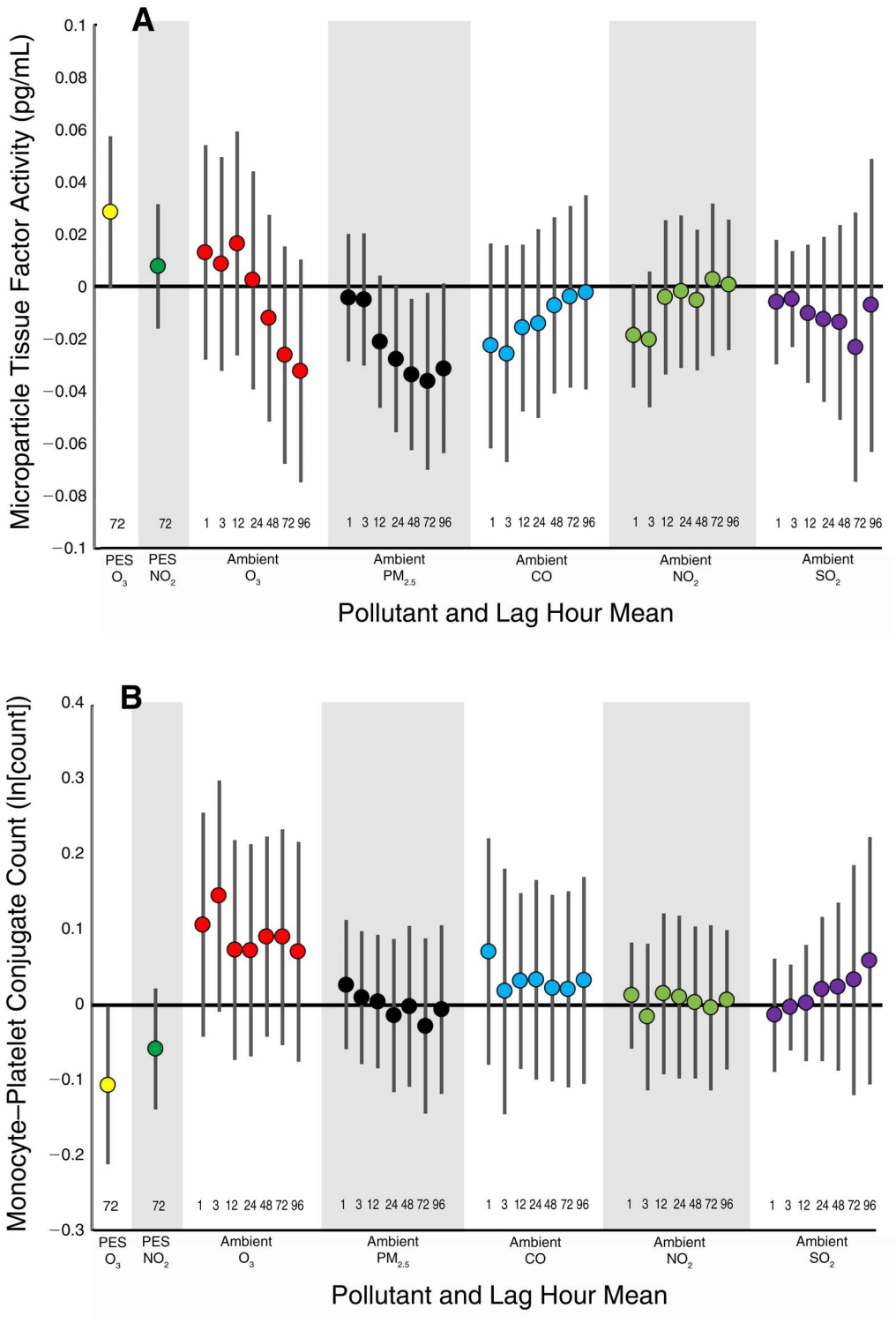

Figure 15. Change in pre-exposure markers of prothrombotic vascular state associated with each IQR increase in personal or ambient pre-exposure pollutant concentration, by pollutant and lag hour mean: (A) microparticle tissue factor activity (pg/mL)and **(B)** monocyte–platelet conjugate count (ln[count]).

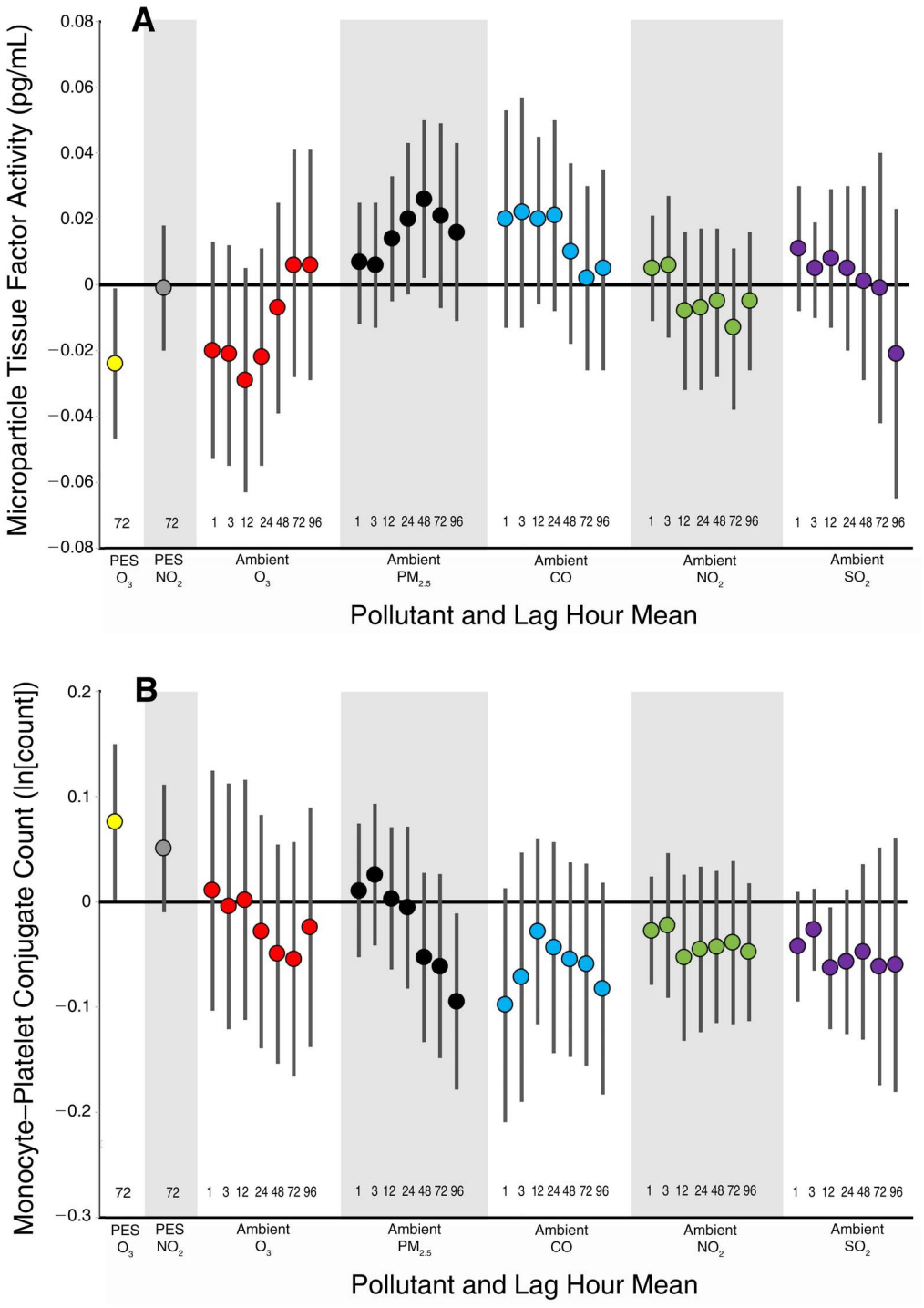

Figure 16. Difference in the pre- to post-exposure change in markers of prothrombotic vascular state associated with each IQR increase in personal or ambient pre-exposure pollutant concentration, by pollutant and lag hour mean: (A) microparticle tissue factor activity (pg/mL) and **(B)** monocyte–platelet conjugate (ln[count]).

exposure session was associated with each 4.1-ppb increase in PES O_3 concentration in the 72 hours before the pre-exposure visit (0.076 ln[count]; 95% CI, 0.002 to 0.150; $P = 0.045$) (Figure 16B; Additional Materials 1, Table 5). Decreased monocyte–platelet conjugate count across the exposure session was marginally significantly associated with each 4.3-μg/m^3 increase in ambient $PM_{2.5}$ concentration in the 96 hours before the pre-exposure visit (−0.094 ln[count]; 95% CI, −0.178 to −0.011; $P = 0.027$) and each 0.8-ppb increase in ambient SO_2 concentration in the 12 hours before the pre-exposure visit (−0.063 ln[count]; 95% CI, −0.121 to −0.005; $P = 0.033$).

Summary of Aim 3 and Aim 4 Findings These findings do not consistently support an association between prothrombotic biomarkers and increases in ambient and personal pollutant concentrations in the 96 hours before the pre-exposure visit.

Pulmonary Function

Aim 3 Decreased pre-exposure FEV_1 was significantly associated with each IQR increase in concentrations of $PM_{2.5}$ in the 1 hour before the pre-exposure visit (−0.022 L; 95% CI, −0.037 to −0.006; $P = 0.007$), CO in the 3 hours before the pre-exposure visit (−0.046 L; 95% CI, −0.076 to −0.016; $P = 0.003$), and NO_2 in the 72 hours before the pre-exposure visit (−0.030 L; 95% CI, −0.052 to −0.008; $P = 0.007$) (Figure 17A; Additional Materials 1, Table 4). With CO the effects were largest with the two earliest lag times and with $PM_{2.5}$ the effect size was similar for all lag times. Decreased FEV_1 was not associated with ambient O_3 or SO_2, or PES O_3 or NO_2. FVC showed similar associations, with patterns of decreased pre-exposure FVC associated with increased $PM_{2.5}$, CO, and NO_2 at most lag times. However, only NO_2 was significant, with each 5.2-ppb increase in NO_2 concentration in the 72 hours before the pre-exposure visit associated with a decrease in FVC of 0.033 L (95% CI, −0.057 to −0.008; $P = 0.009$) (Figure 17B; Additional Materials 1, Table 5).

Aim 4 Increased FEV_1 across the exposure sessions was marginally significantly associated with each 4.1-ppb increase in PES O_3 concentration (0.010 L; 95% CI, 0.004 to 0.026; $P = 0.010$) (Figure 18A; Additional Materials 1, Table 5). Increased FEV_1 across the exposure sessions was also associated with IQR increased $PM_{2.5}$ and CO at all lag times. Increased FEV_1 was significantly associated with increased $PM_{2.5}$ concentrations at multiple lag times (1, 24, 48, 72 hours before the pre-exposure visit), with the largest FEV_1 increase associated with each 4.7-μg/m^3 increase in $PM_{2.5}$ concentration in the 72 hours before the pre-exposure visit (0.018 L; 95% CI, 0.005 to 0.031; $P = 0.007$). Again, patterns of associations were similar for FVC and $PM_{2.5}$, with the largest FVC increase associated with each 4.9-μg/m^3 increase in $PM_{2.5}$ concentration in the 24 hours before the pre-exposure visit (0.015 L; 95% CI, 0.001 to 0.029; $P = 0.039$), with similar sized FVC increases observed for almost all lags (Figure 18B; Additional Materials 1, Table 5). However, there were no associations between changes in FVC across the exposure session and any other pollutant.

Summary of Aim 3 and Aim 4 Findings For Aim 3, increases in ambient concentrations of $PM_{2.5}$, CO, and NO_2 in the 96 hours before the pre-exposure visit were significantly associated with decrements in FEV_1, and perhaps FVC. For Aim 4, the same increases in ambient $PM_{2.5}$ were associated with increases in FEV_1 over the subsequent hours of the exposure sessions. This suggests a "recovery" from the effects of these pollutants during the time in the exposure chamber and laboratory.

Airway Inflammation

Airway inflammation was measured by enumerating inflammatory cells (PMN) in induced sputum, which was performed only at approximately 24 hours after exposure; there was no pre-exposure measurement. Using the same analytic model as for the Aim 4 results described above, with this post-exposure measure of PMN % there were no clear patterns of sputum PMN % association with any pollutant and no statistically significant associations with any pollutant (Figure 19; Additional Materials 2, Appendix G).

Lung Injury

Aim 3 The primary marker of lung injury was levels of club cell protein 16 (CC16). We found no clear pattern of association and no significant associations between increased pollutant concentrations and CC16 at the pre-exposure visit (Additional Materials 1, Table 4).

Aim 4 Increases in $PM_{2.5}$ and CO at multiple lag times were associated with decreases in CC16 across the exposure sessions, with the largest CC16 decreases associated with each 5.4-μg/m^3 increase in $PM_{2.5}$ concentration in the 12 hours before the pre-exposure visit (−0.72 ng/mL; 95% CI, −1.24 to −0.21; $P = 0.006$) and each 0.126-ppm increase in ambient CO concentration in the 1 hour before the pre-exposure visit (−0.90 ng/mL; 95% CI, −1.76 to −0.04; $P = 0.041$) (Additional Materials 3, Appendix F, Figure S8; Additional Materials 1, Table 5;). However, there were no clear associations with other pollutants across lag times.

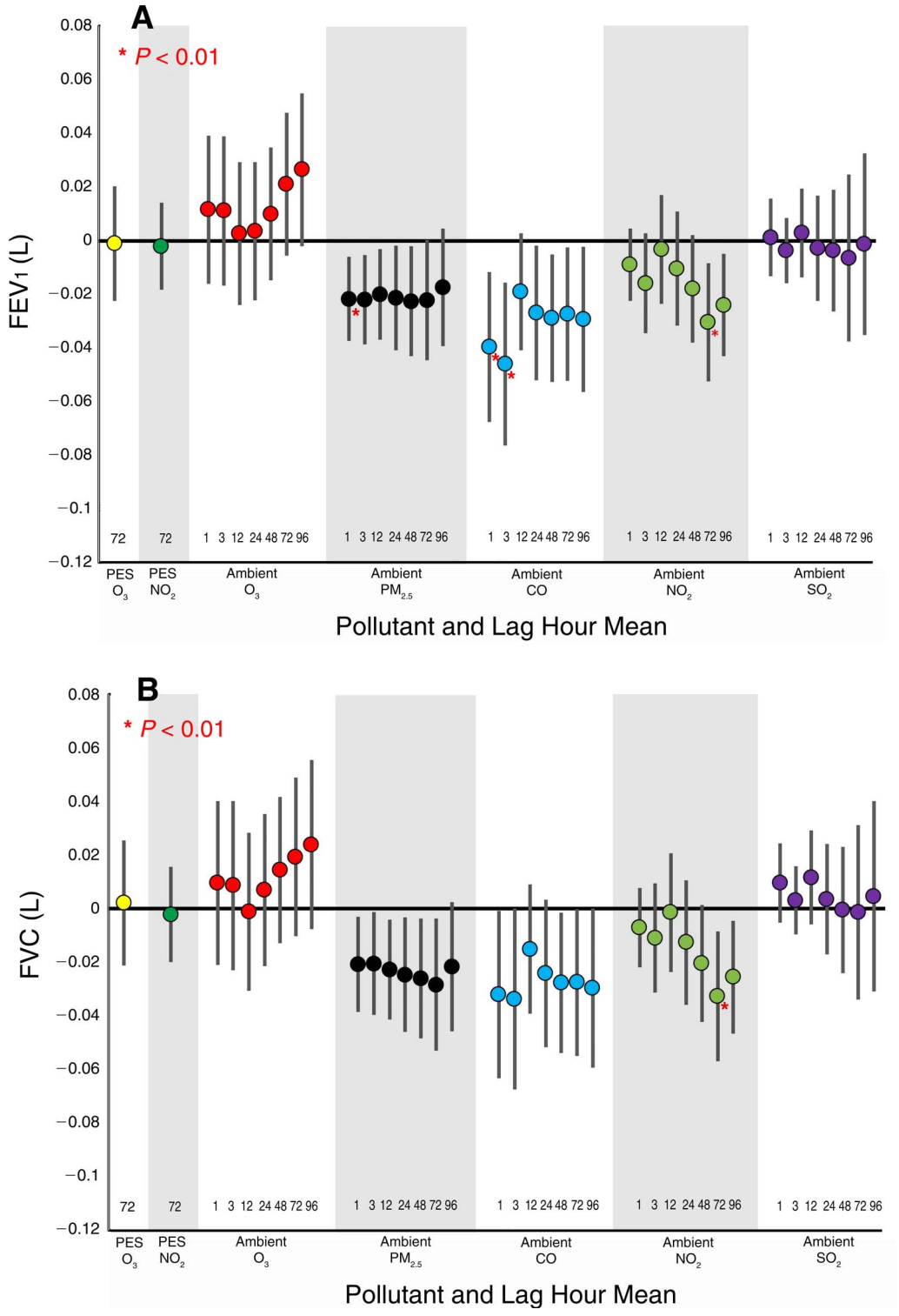

Figure 17. Change in pre-exposure lung function measures associated with each IQR increase in personal or ambient pre-exposure pollutant concentration, by pollutant and lag hour mean: (A) FEV_1 ($P < 0.01$) and **(B)** FVC ($P < 0.01$).

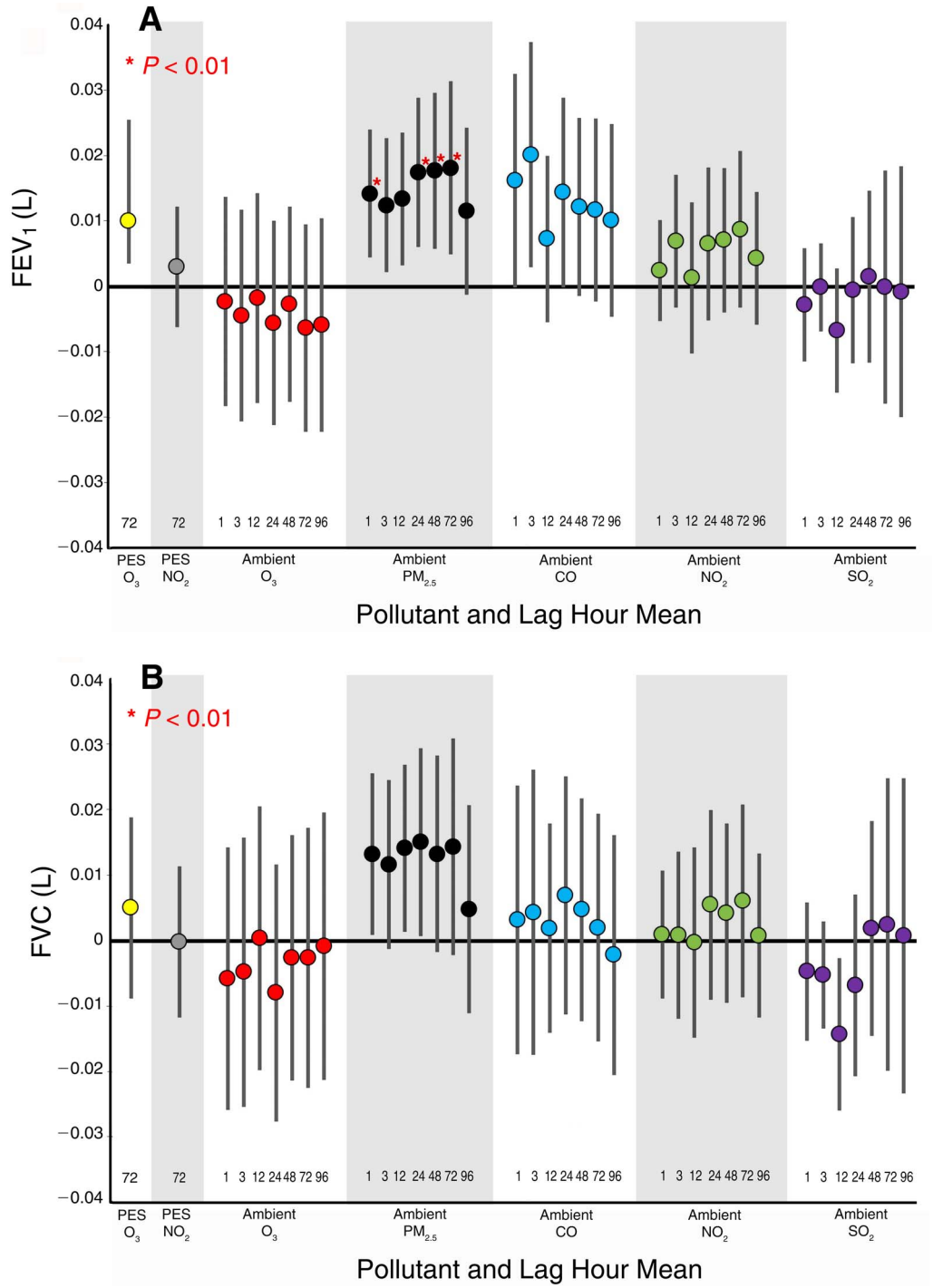

Figure 18. Difference in the pre- to post-exposure change in lung function measures associated with each IQR increase in personal or ambient pre-exposure pollutant concentration, by pollutant and lag hour mean: (A) FEV_1 ($P < 0.01$) and **(B)** FVC.

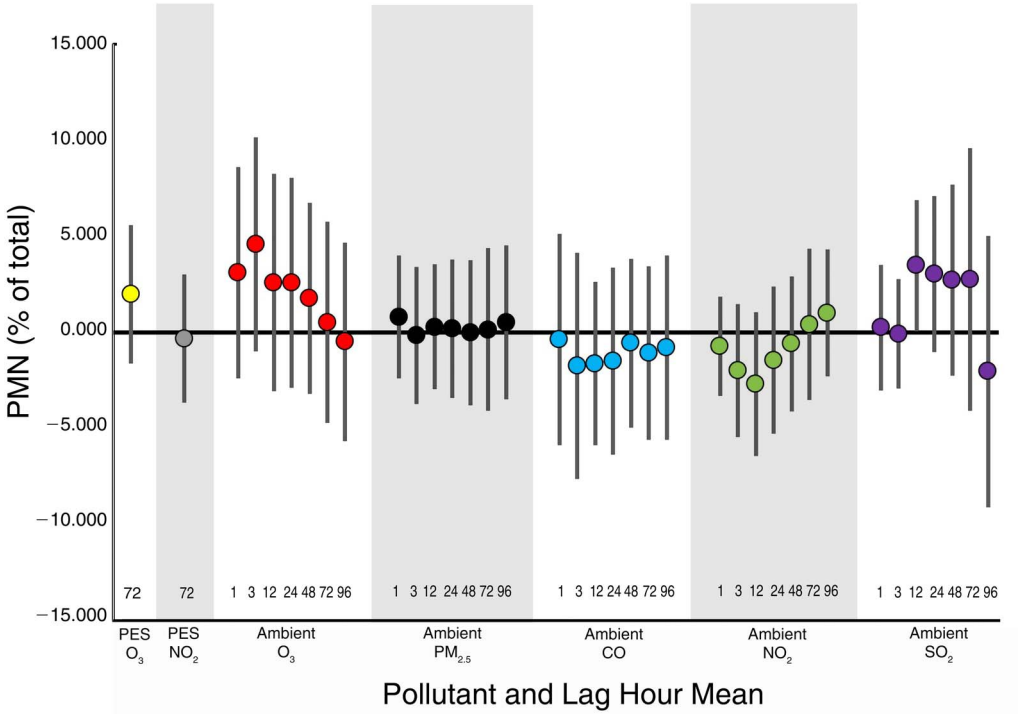

Figure 19. Change in PMN (% of total) associated with each IQR increase in personal or ambient pre-exposure pollutant concentration, by pollutant and lag hour mean.

Summary of Aim 3 and Aim 4 Findings There were no significant effects of prior exposures on baseline CC16. Decreased CC16 across the exposure sessions was significantly associated with increased $PM_{2.5}$ concentration, but the absence of a $PM_{2.5}$-associated increase in CC16 at the pre-exposure measurement in Aim 3 makes the $PM_{2.5}$ findings inconclusive for this outcome.

SENSITIVITY ANALYSES

First, we assessed whether Aim 3 results were substantially different for those outcomes we log transformed in MOSES 2, but did not transform in MOSES 1 (HF, LF, T-wave amplitude, CRP, monocyte–platelet conjugate count, and CC16). There were no substantial differences in our conclusions on effect modification of our controlled O_3 exposure effects on HF, LF, T-wave amplitude, CRP, monocyte–platelet conjugate count, and CC16 when not log transforming each outcome (Additional Materials 2), compared with our main analysis described above, where we did log transform them (Additional Materials 1, Table 4). However, when not log transforming nitrotyrosine, most models did not converge. Next, when not adjusting for relative humidity (Additional Materials 2, Appendix I), our Aim 3 effect estimates were not substantially different, and the inference was generally the same as in our main analysis (Additional Materials 1, Table 4). For example, the change in HF associated with each 10.3-ppb increase in ambient O_3 in the 96 hours before the pre-exposure visit (−0.362 ln[ms^2]; 95% CI, −0.607 to −0.117; P = 0.004) (Additional Materials 2, Appendix I) was smaller, but the inference was the same when not adjusting for relative humidity, compared with our main analysis where we did adjust for relative humidity (−0.460 ln[ms^2]; 95% CI, −0.743 to −0.177; P = 0.002) (Additional Materials 1, Table 4). Last, since we found effect modification by ambient pollutant concentrations, we also calculated the proportion of study subjects from each clinical center, within each tertile for each pollutant. As shown in Additional Materials 1, Table 5, for CO and NO_2, subjects in the High

tertile were predominantly from UCSF (82% and 53%, respectively), whereas subjects in the Low tertile were only from URMC and UCSF. Similarly, for PES NO_2, 50% of study subjects in the High tertile were from UCSF, whereas for subjects in the Low tertile, 69% were from UNC, 25% from URMC, and only 6% from UCSF. For the other pollutants, subjects within each tertile were more equally distributed.

DISCUSSION

In the MOSES 1 (Frampton et al. 2017), we examined whether short-term controlled exposure of older, healthy individuals to peak levels of O_3 regularly experienced outdoors (120 ppb and 70 ppb) induced acute changes in cardiovascular biomarkers compared with exposures to filtered air with 0 ppb O_3. Overall in MOSES 1, we found no significant effects of O_3 exposure on any of the primary or secondary endpoints for autonomic function, repolarization, ST-segment change, arrhythmia, or prothrombotic vascular status. O_3 exposure also did not cause significant changes in the primary endpoints for systemic inflammation (CRP) and vascular function (SBP and FMD) or secondary endpoints for systemic inflammation and oxidative stress (IL-6, P-selectin, and 8-isoprostane). O_3 did cause a significant increase in plasma ET-1 and a marginally significant decrease in nitrotyrosine ($P = 0.017$); plasma ET-1 and nitrotyrosine are secondary markers of systemic oxidative stress and vascular function. The decrease in nitrotyrosine was in the opposite direction of that hypothesized. However, as discussed in the MOSES 1 report, homeostatic responses to increased oxidative stress, including upregulation of antioxidant enzymes, could lead to paradoxical decreases in some markers. However, the MOSES 1 finding with nitrotyrosine may have been spurious, in that it might be related to an increase following 0 ppb O_3, with no change following 70 and 120 ppb O_3. We also found an increase in FVC and FEV_1 after exposure to 0 ppb O_3, which was likely due to the effects of exercise. FEV_1 increased significantly 15 minutes after 0 ppb exposure (85 mL; 95% CI, 64 to 106; $P < 0.001$), and remained significantly increased from pre-exposure at 22 hours (45 mL; 95% CI, 26 to 64; $P < 0.001$). The increase in FVC followed a similar pattern. The increases in FEV_1 and FVC were attenuated in a dose–response manner by exposure to 70 and 120 ppb O_3. We also observed a significant O_3-induced increase in the percentage of sputum PMN 22 hours after exposure at 120 ppb O_3 compared with 0 ppb exposure ($P = 0.003$). Plasma CC16 also increased significantly after exposure to 120 ppb O_3 ($P < 0.001$). However, sputum IL-6, IL-8, and TNF-α concentrations were not significantly different after O_3 exposure. We found no significant interactions with sex, age, or GSTM1 status regarding the effect of O_3 on lung function, percentage of sputum PMN, or plasma CC16. These effects were seen at all three clinical sites. A complete description of MOSES including its protocol and findings are provided in the final report of MOSES 1 (Frampton et al. 2017) and elsewhere (Arjomandi et al. 2018; Balmes et al. 2019; Rich et al. 2018).

In MOSES 2, we used the same linear mixed-effects models used in MOSES 1, coupling data from MOSES 1 with ambient and PES air pollutant concentrations and weather measurements from sites nearby each MOSES clinical site in the 96 hours before the pre-exposure visit. We then examined whether these PES and ambient O_3 and other pollutant concentrations confounded the MOSES 1 findings above (Aim 1). We also examined whether they modified the MOSES 1 controlled O_3 exposure effects on the pre- to post-exposure biomarker changes (Aim 2), and whether these pollutant concentrations were associated with adverse changes in pre-exposure biomarker levels (Aim 3) and pre- to post-exposure changes in these biomarkers, independent of the experimental O_3 exposures (Aim 4). In interpreting the results for each outcome group and Aim, we considered consistency across primary and secondary endpoints, direction of change consistent with that hypothesized, concentration–response relationships, and plausibility. Although an individual effect estimate may have been statistically significant, these other considerations were necessary for us to make a conclusion of effect modification by a pollutant on an outcome group/controlled O_3 association (Aim 2), a conclusion of an association between a pollutant and a pre-exposure outcome group (Aim 3), or a conclusion of an association between a pollutant and a pre- to post-exposure change in an outcome group (Aim 4).

AIM 1

First, we assessed whether the MOSES 1 controlled O_3 exposure effects on cardiovascular and pulmonary biomarkers were confounded by ambient and/or PES pollutant concentrations in the 96 hours before the pre-exposure visit. However, addition of the ambient and PES pollutant concentrations did not substantially alter the MOSES 1 results, and for each outcome the inference remained the same. Thus, ambient and PES pollutants did not appear to confound these previously reported effects of the randomized controlled O_3 exposures on cardiovascular and pulmonary biomarkers (Frampton et al. 2017).

AIM 2

Next, we assessed whether the conclusion of no adverse cardiovascular effects of controlled O_3 exposure, which was made in the final report of MOSES 1, held after taking into account the PES and ambient air pollutant concentrations in the 96 hours before the pre-exposure visit. A priori, we hypothesized that the MOSES controlled O_3 exposures would only cause adverse pre- to post-exposure biomarker changes in response to the controlled O_3 exposures when the PES O_3, PES NO_2, or ambient air pollutant concentrations in the 1 to 96 hours before the pre-exposure visit were low. In other words, any O_3-induced pre- to post-exposure change in a biomarker (e.g., decreased FEV_1 in response to controlled O_3 exposure) would only occur if the PES O_3 concentration was in the lowest tertile of all subjects' PES O_3 concentrations. Otherwise, if the PES O_3 concentration was high (e.g., in the highest tertile of all subjects' PES O_3 concentration), it would block or lessen any adverse effect of controlled O_3 exposures on these outcomes (i.e., no change in FEV_1 in response to controlled O_3 exposure; Hypothesis #1). However, another scenario was also possible. The controlled O_3 exposures may only have had adverse effects on pre- to post-exposure biomarker changes if the PES O_3, PES NO_2, or ambient air pollutant concentrations in the 1 to 96 hours before the pre-exposure visit were high (i.e., in the highest tertile; Hypothesis #2).

Our findings suggest that prior pollutant exposure did not alter the chamber O_3 effects on markers of HRV, repolarization, ST segment, vascular function, oxidative stress, prothrombotic state, or systemic inflammation. However, we did find evidence that ambient NO_2 and CO and PES NO_2 modified the pulmonary function response to controlled O_3 exposures in a concentration–response function. The MOSES 1 finding that chamber O_3 exposures reduced pulmonary function (or mitigated the increase in lung function seen with 0 ppb O_3) was affected by prior pollutant exposures. Consistent with Hypothesis #2, these relatively low levels of experimental O_3 exposure (120 ppb and 70 ppb) reduced pulmonary function only when ambient levels of CO and NO_2, and PES NO_2, were in the highest two tertiles (Figures 6A and 6B; Additional Materials 1, Table 3). This suggests that prior exposures to NO_2 and CO may enhance airway responsiveness to O_3. Thus, although our conclusion in MOSES 1 (that chamber O_3 exposure reduced FEV_1 and FVC) did not change, these Aim 2 findings suggest that increased prior exposures to ambient NO_2 and CO enhanced O_3 effects on lung function, and in fact those exposures may have been required to elicit the experimental O_3 effects. It is also of interest that prior increases in ambient O_3 or PES O_3 did not modify chamber O_3 effects on lung function, given that previous studies have shown pulmonary function effects are attenuated with repeated O_3 exposures (Gong et al. 1997a).

The mechanisms by which prior traffic exposure could enhance airway responsiveness to O_3 are speculative. We did find that increases in these same pollutants were associated with small decreases in pre-exposure FEV_1 and FVC of about the same magnitude (Aim 3, discussed below). It is possible that the enhanced response was caused by reduced airway caliber prior to O_3 exposure. Another possibility is that prior exposures in some way "primed" airway C-fibers, perhaps via sensitization of transient receptor potential A1 cation channels (Bromberg 2016). In addition, the observed effect sizes for the associations were generally small and not likely to be clinically important, especially for people without severe respiratory or cardiovascular disease. However, the importance of these observations lies in demonstrating effect pathways for ambient pollutant exposures and evidence for effect modification in a clinical study by prior ambient exposures.

It should be noted though, that for CO and NO_2, subjects in the High tertile were predominantly from UCSF (82% and 53%, respectively), whereas subjects in the Low tertiles were *only* from URMC and UNC (Table 8). As shown in Table 3, hourly CO and NO_2 concentrations were markedly higher in San Francisco than in Rochester or Chapel Hill. Across all subjects and centers, the ranges of CO concentrations were −0.05 ppm to 0.17 ppm in the Low tertile, 0.17 ppm to 0.25 ppm in the Medium tertile, and 0.26 ppm to 0.75 ppm in the High tertile. The ranges of NO_2 concentrations were −2.2 ppb to 3.4 ppb in the Low tertile, 3.5 ppb to 6.4 ppb in the Medium tertile, and 6.4 ppb to 45.3 ppb in the High tertile. Thus, it is possible that differences in study subject characteristics, slight differences in protocol, or other exposures correlated with ambient CO and NO_2 in San Francisco may, in part, explain why we see this pattern of effect modification of the controlled O_3/pulmonary function response with ambient CO and NO_2. Therefore, caution is warranted in making strong conclusions about these findings and future study is needed to confirm them.

AIM 3 AND AIM 4

For Aim 3, we expected to see that increased personal and ambient pollutant concentrations in the 72 and 96 hours before the pre-exposure visit would be associated with adverse changes in the pre-exposure biomarker level. For Aim 4, we expected to see that the same increased personal and ambient pollutant concentrations would be associated with adverse effects on pre- to post-exposure biomarker changes (e.g., increased pollutant concentrations associated with a decrease in HF from pre- to post-exposure, independent of the controlled O_3 exposure).

Table 8. Proportion of Study Subjects from Each Center, within Each Pollutant Tertile

Pollutant	Tertile	URMC (%)	UNC (%)	UCSF[a] (%)
PES O_3	0	46	28	27
	1	26	38	36
	2	39	36	25
PES NO_2	0	25	69	6
	1	48	21	31
	2	38	12	50
Ambient O_3	0	33	21	46
	1	42	27	31
	2	39	47	15
Ambient $PM_{2.5}$	0	51	28	20
	1	34	33	33
	2	33	27	40
Ambient CO	0	45	55	0
	1	57	33	11
	2	14	4	82
Ambient NO_2	0	49	51	0
	1	36	23	41
	2	29	18	53
Ambient SO_2	0	32	68	—
	1	69	31	—
	2	76	24	—

[a] There were no SO_2 measurements available for subjects from UCSF.

However, our Aim 3 and Aim 4 results for some of the outcomes suggested an unexpected interplay between these Aims. The effects of pollutants on pre-exposure biomarkers in Aim 3 may have affected the pollutant-associated pre- to post-exposure changes in Aim 4. For example, increases in ambient O_3 concentrations in the preceding hours and days were associated with reduced HF HRV at the pre-exposure measurement (Aim 3; in the direction hypothesized). However, HF increased from pre- to post-exposure (a "recovery") in association with increased ambient O_3 (Aim 4; opposite direction from that hypothesized). LF and the secondary HRV outcomes (RMSSD and SDNN) also followed these same patterns. Further, these decreases in HF associated with increased ambient O_3 concentrations appeared independent of other pollutants, as they were little changed when adjusting for other pollutants. However, these Aim 3 changes in pre-exposure HRV associated with increased ambient O_3 concentrations were observed in the previous 72 and 96 hours, but not at shorter time lags (i.e., previous 1–24 hours). Although the timing of this association is inconsistent with our previous work examining $PM_{2.5}$ and ultrafine-particle effects on HRV responses (Rich et al. 2016), it is possible that any ambient O_3 effects on HRV were indirect and involved intermediary processes, such as systemic inflammation (which may take a few days to develop).

Changes in the LF/HF ratio did not achieve statistical significance, reflecting the changes in HF and LF in the same direction in association with ambient O_3. LF/HF ratio is often considered a measure of the balance between the sympathetic and parasympathetic nervous systems. However, the relationship between these two components of the autonomic nervous system is dynamic and complex,

and LF/HF ratio does not always reflect autonomic balance (Shaffer and Ginsberg 2017). LF reflects both sympathetic and parasympathetic influences, as well as other inputs. In the resting state, LF is influenced more by baroreflex activity than sympathetic input. Further, increasing age and heart disease, such as coronary artery disease, heart failure, and myocardial infarction, generally reduce overall HRV, including both LF and HF (Sherazi et al. 2015; Yeragani et al. 1997). Thus we considered the observed reduction in pre-exposure HF and time-domain HRV variables in association with ambient O_3 to be potentially adverse, even without significant changes in LF/HF ratio.

We also observed a similar pattern across Aim 3 and Aim 4 for markers of pulmonary function. Increases in $PM_{2.5}$, CO, and ambient NO_2 concentrations, but not O_3, were associated with decreases in pre-exposure FEV_1 (Aim 3). Again, FEV_1 appeared to recover during the exposure sessions (Aim 4). It is important to recall that in MOSES 1, FEV_1 actually increased across the exposure sessions, and the controlled O_3 exposure attenuated that increase in a concentration–response fashion. FVC followed a similar pattern (Arjomandi et al. 2018; Frampton et al. 2017).

We speculate that the "recovery" of pollutant effects on HRV (following increases in ambient O_3) and pulmonary function (following increases in ambient $PM_{2.5}$, NO_2, and CO) was related to reduced pollutant exposures during the time spent in controlled indoor environments as part of the exposure sessions. For example, subjects spent the night prior to exposure in a local hotel, and then 8 to 9 hours in the research laboratory, before returning home. This included 3 hours in the exposure chamber, breathing air that was both HEPA and Purafil-filtered (with O_3 added per study protocol). During the time in the laboratory, the subjects remained indoors without open windows or exposure to traffic, and without indoor pollutant sources that occur in the home (such as cooking, candles, or fireplaces). Previous studies have shown improvement in markers of cardiovascular function with relatively short-term indoor air filtration (Bräuner et al. 2008; Chen et al. 2015). It is important to note that pre-exposure measurements of HRV and spirometry took place on the morning of the day of exposure, whereas the baseline measures of FMD and other biomarkers took place the day before exposure (i.e., pre-exposure visit). It is therefore possible that pre-exposure HRV and lung function had already "recovered" to some degree from prior ambient exposure effects.

We found no convincing pollutant effects on markers of all the other outcome groups. These included markers of cardiac repolarization, ST segment, vascular function, nitrotyrosine as a measure of oxidative stress, prothrombotic state, systemic inflammation, lung injury, and sputum PMN.

LESSONS LEARNED FROM MOSES 2 AND MOSES 1 FINDINGS

In addition to the conclusions that the MOSES 1 findings were not confounded by PES or ambient air pollutants (Aim 1) and that these prior pollutant exposure did not alter or modify the chamber O_3 effects on any marker (other than pulmonary function [Aim 2]), there are also several lessons learned in our MOSES 2 analyses that should be considered when making inferences from MOSES. First, in order to detect an acute effect of a controlled pollutant exposure (e.g., the 120 ppb and 70 ppb O_3 exposures used in MOSES) on a given biomarker, human controlled exposure studies must provide a contrast between the experimental exposure and subjects' prior ambient air pollution exposures. For studies of short-term pollutant exposures and health responses (e.g., post-exposure measures ≤24 hours of exposure session), in order to see an effect of a controlled pollutant exposure, the level of that exposure has to be substantially greater than the ambient pollutant concentration to which the individual subject is regularly exposed. This could be potentially more of a problem with the lower experimental O_3 concentrations used in MOSES (120 ppb and 70 ppb), which were much closer to the background ambient concentrations to which the subject was exposed in the days before the exposure session (UCSF 1 hour median: 25.9 ppb; UNC median: 35.6 ppb; URMC median: 33.5 ppb), than in past studies, which had much higher controlled O_3 exposure levels (Arjomandi et al. 2015: 200 ppb; Barath et al. 2013: 300 ppb; Devlin et al. 2012: 300 ppb). Of note, all three of these studies with higher controlled O_3 exposure concentrations — and likely a greater contrast between ambient and controlled O_3 exposure concentrations — did report adverse HRV responses to the O_3 exposure. On the other hand, using higher concentrations may limit the relevance of the findings for real-world exposures and for informing air quality standards. This is a concern for all human clinical air pollution studies, and selection of exposure concentrations represents a balance. Thus, future assessment of whether a controlled pollutant exposure has an acute effect on a biomarker must be done while also considering whether the ambient air pollutant concentrations in the previous few days were high or low and what effect they had on the biomarkers under study.

Second, we cannot exclude the possibility of delayed cardiovascular effects of the controlled O_3 exposure, beyond the last measurement in our study (22 hours after exposure). Given that we observed decreased HF HRV

associated with increased ambient O_3 concentrations in the 48 to 96 hours before the pre-exposure visit (i.e., 68 to 116 hours later), it could be that our 22 hours post-exposure biomarker measurement missed a delayed effect of that controlled O_3 exposure. Further, it may be that prolonged exposure to elevated ambient O_3 (e.g., for 5 days or 96 hours) is needed to cause a reduction in HRV.

Third, MOSES 1 was designed to test the effects of O_3 (and only O_3) under controlled laboratory conditions in the absence of other pollutants. However, we found associations between markers of HRV and ambient O_3 concentrations in MOSES 2, but not the controlled O_3 exposures of MOSES 1. It is possible that other pollutants in ambient air could generate secondary reaction products or, in some other way, exert synergistic, potentiating, or attenuating cardiovascular effects in combination with O_3. Thus, although the O_3 exposure in the chamber was just O_3, the effect of ambient O_3 is likely also reflecting the effects of other oxidants existing in the air pollution milieu with O_3. This may also help explain the difference in MOSES 1 (controlled O_3) and MOSES 2 (ambient O_3) findings for HRV.

HEART RATE VARIABILITY: COMPARISON TO OTHER STUDIES

As discussed in the final report of MOSES 1 (Frampton et al. 2017), several controlled exposure studies examined HRV responses to short-term O_3 exposures (Arjomandi et al. 2015; Barath et al. 2013; Devlin et al. 2012; Fakhri et al. 2009; Sivagangabalan et al. 2011), with conflicting findings. Briefly, Sivagangabalan and colleagues found no HRV response to exposure to 120 ppb compared with 0 ppb O_3 for 2 hours at rest, whereas Fakhri and colleagues reported a 467-ms^2 increase in HF HRV following a 120 ppb O_3 exposure. Barath and colleagues found no statistically significant effect of a 300 ppb O_3 exposure for 75 minutes (with exercise) on any metric of HRV, but normalized HF increased from 13 to 24 ms^2 2 hours after exposure to the same 300 ppb O_3 exposure while at rest. Arjomandi and colleagues reported a significant increase in mean normalized LF (and a reciprocal decrease in mean normalized HF) 20 hours after exposure, based on regression modeling of combined data from 4-hour exposures to 0 ppb, 100 ppb, and 200 ppb O_3 in 26 young nonsmokers, 10 of whom had mild asthma. In that study, the major difference in HRV parameters among the three exposures may have been regression to the mean, related to differences in the pre-exposure baselines (e.g., normalized LF at 0 ppb: 54.4; at 100 ppb: 49.1; at 200 ppb: 46.6) rather than the post-exposure measures (e.g., normalized LF 24 hours post-exposure at 0 ppb: 51.5; at 100 ppb: 52.0; at 200 ppb: 51.6). There was very little difference in the pre- versus immediate post-exposure values of SDNN for any of the exposures. Devlin and colleagues (2012) exposed healthy young nonsmokers to 300 ppb O_3 for 2 hours with intermittent moderate exercise, and reported a 41% decrease in HF 1 hour after O_3 exposure (pre-exposure: 3,132 ms^2; 1 hour after: 1,833 ms^2). However, LF also fell substantially immediately after O_3 exposure (−36%), so that their reported mean LF/HF ratio values post-exposure were similar for air and O_3. Together, these controlled O_3 exposure studies do not provide clear evidence of an O_3-provoked increase in autonomic sympathetic tone. Further, our Aim 3 findings of decreased HF, LF, RMSSD, and SDNN associated with increased ambient O_3 concentrations in the 48 to 96 hours prior to the chamber exposure are consistent with these controlled O_3 exposure study findings (but not the MOSES 1 findings) where there was no HRV response to the controlled O_3 exposure after 24 hours (Frampton et al. 2017; Rich et al. 2018). This may simply reflect that the ambient O_3 is a surrogate of photochemical smog and reflects the total oxidative potential of air pollution, not just its own oxidative potential. This is discussed further in *Air Pollution Considerations* section below.

Several observational panel studies have examined acute HRV responses to ambient O_3 concentrations with mixed findings and have been reviewed previously (Buteau and Goldberg 2015; U.S. EPA 2019). The U.S. EPA Integrated Science Assessment (2013) concluded there was inconsistent evidence of an HRV/O_3 association across these studies. They concluded that analyses done using the Veterans Affairs Normative Aging Study (Park et al. 2005) reported decreases in HRV markers associated with increased ambient O_3 concentrations, but the majority of other studies reported no association (U.S. EPA 2013). Buteau and Goldberg (2015) reviewed many of these same studies, but reported that there were too few high quality studies to make any conclusions, based on their argument that many papers' analyses inappropriately underestimated standard errors.

The populations included in these O_3 panel studies were diverse. Some studies included older subjects only (Bartell et al. 2013: ≥71 years; Holguin et al. 2003: 60–96 years; Jia et al. 2011: 52–73 years; Mirowsky et al. 2017: 40–75 years), and others included younger subjects only (Chuang et al. 2007: 18–26 years; Shields et al. 2013: 22–56 years; Wu et al. 2010: 25–46 years). Some studied subjects with pre-existing coronary artery disease (Bartell et al. 2013; Lipsett et al. 2006; Mirowsky et al. 2017; Zanobetti et al. 2010); those with ischemic heart disease, hypertension, or diabetes (Park et al. 2005); or those with either a prior myocardial infarction or chronic obstructive pulmonary disease (Suh and Zanobetti 2010; Wheeler et al. 2006).

Our findings are consistent with those studies reporting significant decreases in HRV markers associated with increased O_3 concentrations. Although the time lags of response differ, our findings of decreased HF HRV associated with acute increases in O_3 concentrations in the previous few days in subjects 55–70 years of age, are consistent with Jia and colleagues (2011) who studied nonsmoking, non-drinking, healthy Beijing residents 52–73 years of age, and reported a reduction (−4.87%; 95% CI, −8.62% to −0.97%) in 5-minute average HF associated with each 10-ppb increase in ambient O_3 concentration in the previous 5 minutes. Among young Mexico City residents (22–56 years of age), each 65-ppb increase in O_3 concentration in the previous 90 minutes was associated with a significant 16.48% reduction in HF (Shields et al. 2013). Among young healthy Taipei, Taiwan students, each 12-ppb increase in ambient O_3 concentration in the previous 3 days was associated with a reduction in HF (−6.6%; 95% CI, −11.8% to −1.4%). In elderly nursing home residents in Mexico City, Holguin and colleagues reported a 0.031 $\log_{10}(ms^2)$ reduction in HF associated with each 10-ppb increase in the 1-hour maximum ambient O_3 concentration (Holguin et al. 2003). Our finding of a reduction in HF (−0.460 $\ln(ms^2)$; 95% CI, −0.743 to −0.177) associated with each 10.35-ppb increase in O_3 in the 96 hours before the pre-exposure visit (~ 7.0% decrease in HF; mean pre-exposure HF = 706.6 ms^2; [−0.460 / ln(706.6) × 100%] = −7.0%) is consistent with these studies and of similar size.

However, numerous other studies have reported no significant association between increased ambient O_3 and HF (Mirowsky et al. 2017; Park et al. 2005; Suh and Zanobetti 2010; Wheeler et al. 2006; Wu et al. 2010), although some reported similarly sized, non-statistically significant HF changes associated with increased O_3 concentrations. For example, Mirowsky and colleagues reported a non-significant 21.9% reduction in HF associated with each IQR increase (no actual IQR provided) in mean O_3 concentration in the previous 5 days, among 13 adult patients 40–75 years of age with coronary artery disease (Mirowsky et al. 2017). Park and colleagues, in the Veterans Affairs Normative Aging Study, reported a non-significant decrease in HF (−11.1%; 95% CI, −26.2% to 7.1%) associated with each 13-ppb increase in ambient O_3 concentration in the previous 24 hours, among men aged 21–81 years of age (mean = 71) (Park et al. 2005). These and other studies examined other HRV metrics as well (e.g., RMSSD, SDNN, and LF), and reported similar mixed findings with each marker (Buteau and Goldberg 2015; Park et al. 2005; U.S. EPA 2019). Our findings support the conclusion that ambient O_3 exposure adversely affects HRV in older, healthy subjects.

PULMONARY FUNCTION: COMPARISON TO OTHER STUDIES

Our MOSES 2 analyses confirmed the pulmonary function responses to the experimental O_3 exposures reported for MOSES 1 (Arjomandi et al. 2015; Frampton et al. 2017). The increase in FEV_1 following exposure to 0 ppb was attenuated by increasing O_3 concentrations. Further, as described in the final report of MOSES 1 (Frampton et al. 2017), several previous studies have examined pulmonary function responses to acute O_3 exposure in older healthy volunteers (51–89 years of age), but all used considerably higher O_3 concentrations. These studies used O_3 exposure durations from 1 to 4 hours; some studies included continuous or intermittent exercise at moderate ventilation rates (20–29 L/min). O_3 concentrations ranged from 240 ppb to 450 ppb (Bedi et al. 1988, 1989; Drechsler-Parks et al. 1987, 1989, 1990; Gong et al. 1997a, 1997b; Reisenauer et al. 1988), all of which were markedly higher than the 120 ppb and 70 ppb used in MOSES. Drechsler-Parks and colleagues reported that exposures up to 450 ppb O_3 for 2 hours with intermittent exercise elicited statistically significant decrements in spirometric variables (FVC of −5.3% and FEV_1 of −5.6%) (Drechsler-Parks et al. 1987). In the Gong and colleagues (1997b) study of healthy older men, an O_3 exposure dose roughly equivalent to that of Drechsler-Parks and colleagues (1987) (240 ppb for 4 hours with intermittent exercise) reduced mean FEV_1 by 1.9% (Gong et al. 1997b). Reisenauer and colleagues reported that 1-hour exposure to 200 ppb and 300 ppb O_3 via mouthpiece with moderate intermittent exercise, which was ~25% of the inhaled dose used by Drechsler-Parks and colleagues (1987), did not produce any significant spirometric effects (Reisenauer et al. 1988).

Panel studies have also been used to study acute pulmonary function responses to ambient O_3 (U.S. EPA 2019). Studies in healthy children and in both children and adults with asthma have shown significant decrements in lung function associated with ambient O_3 exposure, especially with physical activity outdoors, such as with children attending summer camp and outdoor workers. These studies (reviewed in U.S. EPA 2019) are generally consistent with clinical studies of O_3 in adults.

However, studies in older healthy adults are inconsistent. Hoppe and colleagues found no significant associations between ambient O_3 concentrations at lag 0–1 days and lung function in 41 subjects 69 to 95 years of age (Hoppe et al. 2003). Alexeeff and colleagues (2007), using spirometry data from the Veterans Affairs Normative Aging

Study (n = 904; mean age, 68.8 yr; SD, 7.3 yr), found significant decreases in FEV_1 associated with prior increases in O_3 exposure, with increased responses in subjects with obesity and/or airway hyper-responsiveness. The same group later studied acute and subchronic epigenetic and lung function effects of O_3 on the Veterans Affairs Normative Aging Study population, but subjects with lung disease were included (Lepeule et al. 2014a, 2014b). They found lung function effects of subchronic exposure to black carbon, CO, and NO_2, but not O_3. Rice and colleagues (2013) studied offspring of the Framingham cohort (n = 3262, mean age = 51.8 years SD 12.7 years, 21% with asthma or COPD). The FEV_1 decreased (−17.4 mL, 95% CI, −30.9 to −4.0) per 10-ppb increase in ambient O_3 at lag days 1–2. Although the lag times differ, our results support Lepeule and colleagues' findings of lung function effects of traffic-related pollutants, but not O_3, in older subjects (Lepeule et al. 2014b).

AIR POLLUTION CONSIDERATIONS

As discussed above, we found that increased concentrations of ambient O_3, but not PES O_3, were associated with decreased HF, LF, RMSSD, and SDNN HRV. This lack of consistency across O_3 measures is not surprising. Measurements of ambient O_3 are a surrogate of photochemical smog and reflects the total oxidative potential of air pollution, not just its own oxidative potential. When ambient O_3 enters the indoor environment, however, it can also be removed by gas-phase reactions (e.g., its reaction with both NO and NO_2) or by heterogeneous reactions taking place on indoor surfaces. This explains why indoor or PES O_3 levels are typically lower than those outdoors. However, both the homogeneous and heterogeneous reactions produce reactive species such as NO_3 (nitrate radical) and CH_2O (formaldehyde) among others. Therefore, the sum of reactive species indoors is related to the total amount of O_3 entering the home, which is correlated with ambient O_3. The correlation between PES O_3 and ambient O_3 may be weakened depending upon the presence of combustion sources and low home air-exchange rates. Further, as shown by our substantially higher ambient O_3 concentrations versus PES O_3 concentrations, indoor O_3 levels are generally lower than outdoor O_3 levels because O_3 is reactive and is removed by indoor surfaces. In addition, in the presence of indoor combustion sources (e.g., gas stoves), O_3 reacts with both NO and NO_2, and thus remains at much lower concentrations indoors than outdoors. O_3 outside the participant homes may also be low when homes are located in areas with high traffic due to scavenging of O_3 by NO and NO_2. Thus, it is not surprising to see different biomarker responses to ambient O_3 and PES O_3.

Of note, we also observed higher concentrations of PES NO_2 than ambient NO_2 at UNC and URMC. PES NO_2 levels can exceed ambient NO_2 concentrations if the study participant lived in areas where the concentrations of NO_2 outside their home was higher than levels measured by the ambient monitor. This can happen when the participant lives in a high traffic area, and/or lives in a home with combustion sources that emit NO and NO_2. For example, natural-gas stoves are an important source of indoor NO and NO_2, and thus, PES NO_2 (in part reflecting indoor NO_2) and ambient NO_2 may largely represent different pollution sources. Thus, as was the case with O_3, it is not surprising to see different biomarker responses to ambient NO_2 and PES NO_2. Last, it is important to note, that the ambient O_3 and other pollutant concentrations at each site were low and regularly below the National Ambient Air Quality Standard levels. It is not clear if the same null findings would occur if this study was repeated in a location with substantially higher ambient O_3 and other pollutant concentrations.

STRENGTHS AND LIMITATIONS

This study had several strengths. MOSES, a multicenter study, was one of the largest controlled human exposure studies of O_3 to be conducted to date, providing greater statistical power than previous studies. It measured primary and secondary markers of the major pathways by which air pollutants may contribute to acute cardiovascular toxicity, assessed both acute cardiovascular and respiratory responses to O_3, and had careful adherence to protocols across the three study centers. Using these health data, ambient air pollutant measurements from nearby monitors, and personally measured O_3 and NO_2 concentrations, we were able to evaluate whether ambient and personally measured pollutant concentrations in the 96 and 72 hours before the pre-exposure visit affected baseline and pre- to post-exposure changes in biomarkers and whether they modified any controlled O_3 exposure effects on those same pre- to post-exposure biomarker changes. Other groups have also applied a repeated-measures panel study approach to examine whether ambient air pollutants in the few hours and days before pre-exposure biomarker measurements, measured as part of a controlled pollutant exposure study, were associated with increases or decreases in those biomarkers (Gandhi et al. 2014; Thompson et al. 2010). To our knowledge, this is the first such controlled O_3 exposure study to also conduct these additional panel study analyses. However, there are also several limitations that should be considered when making inferences. Below, we describe those limitations for each Aim separately.

Aim 1 Our Aim 1 analyses examined whether ambient or PES O_3 and other pollutant concentrations in the 96 hours before the pre-exposure visit confounded the effect of the MOSES controlled O_3 exposure on the pre- to post-exposure change in biomarkers. However, all MOSES subjects were assigned the same ambient pollutant concentrations for a specific hour/day, regardless of how close they lived to the air pollution site, resulting in some mismeasurement of each subject's ambient air pollution exposure. Thus, there may be residual confounding by ambient air pollution exposure remaining in these analyses.

Aim 2 In our Aim 2 analyses — examining whether PES or ambient pollutant exposures or concentrations modified the controlled O_3 exposure effect on the pre- to post-exposure biomarker change — there is also a chance for residual confounding. This potential residual confounding could be due to coding continuous PES exposure or ambient pollutant concentrations into tertiles, which might not entirely control for the effect of PES exposure. In addition, because a subject might not be in the same tertile of PES exposure before each of their three exposure visits, the estimate of the PES by controlled exposure interaction (and the main effect of PES exposure) may be determined by an unbalanced number of observations per subject within each level. This should not be problematic, unless the pattern of PES exposure in association with controlled exposure was different for subjects with large pre- to post-exposure biomarker changes than for subjects with small biomarker changes. In that case this could cause a bias in the estimated exposure effects and possibly a biased estimate of the variance of the random subject-specific effect. Second, it is possible that some subjects may not have worn the PES samplers for all of the 72 hours before each pre-exposure visit. This may have mis-specified those subjects' PES O_3 and/or PES NO_2 exposures in our Aim 1 analyses, resulting in residual confounding. However, the MOSES 1 controlled O_3 effects on each biomarker were not substantially different when controlling for PES or ambient pollutants, suggesting any residual confounding by a mis-specified PES O_3 or NO_2 concentration was minimal.

Third, as in MOSES 1, we cannot generalize our lack of cardiovascular responses to controlled O_3 to populations exposed to higher concentrations. By design, we restricted participation in MOSES to older, healthy subjects, who were physically fit enough to complete the exercise regimen. Thus, our subjects cannot be considered representative of the general population or of all people in this age range. People with pre-existing cardiovascular or pulmonary disease may differ in their responses to ambient O_3 and other pollutants. The choice to study older subjects was based on the hypothesis that these individuals would be most at risk for acute cardiovascular effects of O_3. This may not be the case, however. Younger individuals are known to be more responsive to the effects of O_3 on lung function and may also be more responsive with regard to cardiovascular parameters (McDonnell at al. 2012).

Aim 3 and Aim 4 First, our Aim 3 and Aim 4 analyses were observational, in that they examined the association between ambient and PES pollutant concentrations (measured and not randomly assigned as the controlled O_3 exposures in MOSES 1) and pre-exposure biomarkers or pre- to post-exposure biomarker changes. Thus, although we have controlled for several potential confounders in these analyses, including temperature, relative humidity, site, and time, residual confounding is still possible.

Second, we did not document each subject's residential location relative to the air pollution monitoring site. All MOSES subjects were assigned the same ambient pollutant concentrations for a specific hour/day, regardless of how close they lived to the monitoring site, which likely resulted in exposure misclassification. However, this exposure misclassification or error is likely a combination of Berkson and classical error, resulting in a bias toward the null and underestimates of effect (Bateson et al. 2007; Zeger et al. 2000).

Third, these PES samples provide estimates of each subject's personal NO_2 and O_3 exposure during the 72 hours before each pre-exposure visit. As in Aim 1, we did not have information on the degree of PES monitoring protocol compliance of each study subject (i.e., did they wear the PES for all hours during the 72 hours before the pre-exposure visit?), which may have mis-specified a subject's PES O_3 and/or PES NO_2 exposure. In Aims 3 and 4, this error was likely non-differential, resulting in a bias towards the null and underestimates of effect.

Fourth, similar to Aim 2, we cannot generalize our lack of cardiovascular responses (for all outcomes other than HRV and pulmonary function) to ambient O_3 and other pollutants to populations exposed to higher concentrations.

Fifth, similar to Aim 2, our subjects cannot be considered representative of the general population or of all people in this age range.

Sixth, it is important to note that the pre-exposure measures of some biomarkers were measured during the pre-exposure visit, while some were measured on the morning of the exposure days.

Seventh, it is important to note that the pre-exposure measures of some biomarkers were measured during the pre-exposure visit, while some were measured on the morning of the exposure days. For example, as described

in the section *Brief Description of Original MOSES Study Protocol* and shown in Figure 1, most biomarkers (e.g., systemic inflammation, oxidative stress, prothrombotic vascular status) were measured at the pre-exposure visit (i.e., day before the exposure session). However, all ECG outcomes (e.g., HRV, cardiac repolarization, ST segment) and pulmonary function biomarkers were measured on the morning of the exposure day of each exposure session (~20 hours after pre-exposure visit). Thus, the associations between HRV, repolarization, ST segment, and pulmonary function markers and ambient and PES pollutant concentrations in the 1–96 hours before the pre-exposure visit correspond to lag times between pollutant and biomarker measurement of ~21–116 hours. In contrast, the associations between other biomarkers (e.g., systemic inflammation, oxidative stress, prothrombotic vascular status) and these same pollutants correspond to lag times of 1–96 hours.

Last, for Aim 4 we used the same analytic approach as MOSES 1, a mixed-effects linear regression with the pre- to post-exposure biomarker changes as the outcome in all models. However, one potential disadvantage of using change as the primary outcome measure is the possibility of random baseline differences with regression to the mean for subsequent measurements. This can result in a statistically significant treatment effect that is in fact spurious. The apparent difference in outcome (change from baseline) is caused by a chance difference between the baseline measurements, before the air and O_3 exposures, with post-exposure values closer to the mean, resulting in change in opposite directions for the experimental and control exposures. We addressed this in MOSES 1 by examining baseline data as well as the change data, and by providing the mean baseline data for each of the three exposure conditions in all graphic representations of changes. Differences at baseline were considered in our interpretation, as well as hypothesized direction of change and concentration–response relationships. In this report, we also used coherence of related outcomes in determining whether statistically significant effects could be spurious.

CONCLUSIONS

Coupling data from the Multi-center Ozone Study of oldEr Subjects (MOSES) with ambient and personally measured air pollutant concentrations in the 96 and 72 hours before the pre-exposure visit, we found that these ambient concentrations and personally monitored pollutant exposure did not confound the reported associations between the controlled O_3 exposure and pre- to post-exposure biomarkers. Second, we found that prior ambient and personal O_3 and other pollutant concentrations did not modify or mask the effect of the controlled O_3 exposures on HRV, cardiac repolarization, ST segment, vascular function, nitrotyrosine as a measure of oxidative stress, prothrombotic state, systemic inflammation, lung injury, or sputum PMN. However, increases in ambient concentrations of NO_2, CO, and $PM_{2.5}$ may have modified the pulmonary function response to the controlled O_3 exposure in a concentration–response manner. Lung function effects of chamber O_3 exposures were not seen when concentrations of NO_2, CO, and $PM_{2.5}$ were in the lowest tertile. However, since the distribution of clinical centers within tertiles of CO and NO_2 were markedly different between High and Low tertiles, this needs to be confirmed in future studies.

In our observational analyses, using a longitudinal panel study approach, decreases in pre-exposure HRV were associated with short-term increases in ambient O_3 concentrations and decrements in pre-exposure pulmonary function were associated with increases in CO, NO_2, and $PM_{2.5}$ but not O_3. Other outcomes were not affected. There appeared to be an increase or "recovery" in HRV and pulmonary function during the exposure sessions, possibly related to removal of the subjects from exposure to O_3 and traffic. The lack of concurrence of the observed associations between outcome groups, PES O_3, and ambient O_3 are, in part, explained by gas-phase reactions indoors between O_3 and NO/NO_2 and heterogeneous reactions taking place on indoor surfaces resulting in substantially lower indoor O_3 concentrations, and other oxidants/pollutants correlated with ambient O_3 but not PES O_3. Similarly, the lack of concurrence of observed associations between outcome groups, PES NO_2, and ambient NO_2 can be explained, in part, by differences in indoor and outdoor NO_2 sources and resulting concentrations.

Future controlled exposure studies should consider the impact of ambient pollutants on pre-exposure biomarker levels, and whether they modify any health response to the controlled pollutant exposure. We conclude that, in clinical studies of O_3 exposure, prior ambient pollutant exposures may alter pre-exposure measurements of pulmonary and cardiovascular function and thus affect the results of the experimental exposure.

IMPLICATIONS OF FINDINGS

MOSES was the first multicenter controlled air pollution study and the first to examine effects of short-term O_3 exposure on cardiovascular outcomes in older subjects. In MOSES 1, there were no responses to controlled O_3 exposures (120 ppb and 70 ppb) by markers of several mechanistic

pathways contributing to cardiovascular disease, including cardiac autonomic control, repolarization, and arrhythmia, as well as markers of systemic inflammation, vascular function, oxidative stress, and propensity for thrombosis. However, we did observe reduced FEV_1 and FVC (markers of pulmonary function) in response to the O_3 exposures. In MOSES 2, our findings support the conclusion that ambient O_3 concentrations, and perhaps other oxidants correlated with it, adversely affect HRV in older, healthy subjects. Given that this response to increased concentrations of ambient O_3 was maximal after averaging times of 72 and 96 hours, it is possible that our last post-exposure measurement in MOSES 1 (at 22 hours) missed delayed effects of the controlled O_3 exposures on HRV. Second, our findings support lung function responses to ambient CO, NO_2, and $PM_{2.5}$ concentrations, but not O_3, in older, healthy subjects. Third, our findings that pulmonary function responses to controlled O_3 exposure occurred only when ambient traffic pollutant concentrations in the 72 hours before the preexposure visit were high, suggest that there may have been some sort of physiologic priming caused by these traffic pollutants and that the controlled O_3 exposures used in MOSES by themselves may not have been sufficient to produce a pulmonary function response.

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HEI QUALITY ASSURANCE STATEMENT

The MOSES study underwent quality assurance by an independent auditor, David Bush and Associates, according to HEI's quality assurance guidelines, see the Quality Assurance Statement in MOSES 1 (Frampton et al. 2017). All data used in the MOSES 2 analyses were audited as part of MOSES 1. Statistical modeling approaches in MOSES 2 were similar to the audited statistical analyses used in MOSES 1.

MATERIALS AVAILABLE ON THE HEI WEBSITE

The Additional Materials for this report contain supplemental material not included in the printed report. They are available on the HEI Web site, *www.healtheffects.org/publications*.

Additional Materials 1. Supplementary Tables for Primary Endpoints

Additional Materials 2. Supplementary Tables for Secondary Endpoints.

Additional Materials 3. Supplementary Figures for Secondary Endpoints.

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David Q. Rich, co-principal investigator, received his Sc.D. in epidemiology and environmental health from the Harvard School of Public Health (HSPH) in 2004, and was a post-doctoral fellow at both HSPH and the Division of Aging at Brigham and Women's Hospital from 2004 to 2005. He was an assistant professor at the University of Medicine and Dentistry of New Jersey School of Public Health (now the Rutgers School of Public Health) and Environmental and Occupational Health Sciences Institute from 2005 to 2010. He is an environmental epidemiologist and an associate professor in the Division of Epidemiology, Department of Public Health Sciences, with secondary appointments in the Departments of Medicine and Environmental Medicine at the University of Rochester Medical Center in Rochester, New York. Rich's primary research interests include the cardiopulmonary and reproductive health effects of exposure to air pollution and other environmental toxicants.

Mark W. Frampton, co-principal investigator, received his M.D. from New York University School of Medicine in 1973 and then trained in internal medicine at Buffalo General Hospital, Buffalo, New York. He is a pulmonologist and professor emeritus in the Division of Pulmonary and Critical Care Medicine, Department of Medicine at the University of Rochester Medical Center in Rochester, New York. His primary research interest has been the health effects of air pollution, and he has led numerous human clinical studies examining the cardiopulmonary health effects of short-term air pollutant exposure in healthy subjects, as well as in subjects with asthma and type 2 diabetes.

John R. Balmes received his M.D. from Mount Sinai School of Medicine in 1976. After training in internal medicine at Mount Sinai and pulmonary subspecialty, occupational medicine, and research training at Yale, he joined the faculty of the University of Southern California in 1982. He joined the faculty at the University of California at San Francisco in 1986 and is currently professor and division chief of Occupational and Environmental Medicine at San Francisco General Hospital (SFGH). His major academic activities include his research laboratory, several collaborative epidemiological research projects, and direction of the clinical occupational and environmental medicine division at SFGH. His laboratory, the Human Exposure Laboratory, has been studying the respiratory health effects of various air pollutants for the past 28 years.

Philip A. Bromberg received his M.D. from Harvard Medical School in 1953 and held fellowships at Mount Sinai Hospital, New York City, and at Harvard Medical School, Boston, Massachusetts. In 1971, he was appointed professor of medicine and director of the Division of Pulmonary Diseases, Department of Medicine, Ohio State University, Columbus, Ohio. In 1975 Bromberg became director of the Division of Pulmonary Diseases and professor of medicine in the Department of Medicine, University of North Carolina at Chapel Hill. He is currently scientific director of the Center for Environmental Medicine, Asthma and Lung Biology, University of North Carolina at Chapel Hill. Bromberg is a pulmonary physician and inhalation toxicologist with long experience with controlled exposures of human subjects to ozone, including mechanistic studies of the lung function changes.

Mehrdad Arjomandi received his bachelor's degree in molecular biology from the University of California at San Diego in 1991, and his M.D. from Stanford University School of Medicine in 1996. He completed his residency in internal medicine at the University of California at Los Angeles Medical Center (1999) and his fellowship in pulmonary and critical care medicine at the University of California at San Francisco (UCSF) (2003). Arjomandi is currently associate professor of medicine in the Division of Pulmonary, Critical Care, Allergy, Immunology, and Sleep Medicine at UCSF

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Sally W. Thurston received her Ph.D. in statistics from Harvard University in 1997. She was a postdoctoral fellow and then a research associate in biostatistics at the Harvard School of Public Health from 1997 to 2002. She is now an associate professor of biostatistics and oncology in the Department of Biostatistics and Computational Biology at the University of Rochester Medical Center in Rochester, NewYork. Thurston's collaborations in environmental health include studies of longitudinal effects of prenatal methylmercury exposure and studies of reproductive effects of air pollution. Her statistical work includes methods for multiple outcomes, measurement error, and Bayesian inference. Thurston is the principal investigator of her department's T32 grant "Training in Environmental Health Biostatistics."

Petros Koutrakis received his Ph.D. in environmental sciences from the University of Paris, France, in 1984, and was a post-doctoral fellow at both the Harvard Kennedy School of Government and School of Public Health. He served as assistant and associate professor of Environmental Sciences at the Harvard School of Public Health between 1988 and 1994, and was promoted to professor in 1995. He has served as the editor-in-chief for the *Journal of Air Waste Management Association* for almost ten years. He has expertise in ambient and indoor air quality, sampling and analysis of air pollutants, and exposure assessment. He developed the Ogawa personal O_3 sampler and holds a patent for this method.

Kelly Thevenet-Morrison received her M.S. degree in statistics from Rutgers University, and is the lead programmer analyst in the Department of Public Health Sciences at the University of Rochester Medical Center. Thevenet-Morrison has an extensive background in data management, programming, modeling, and statistical analysis, and has provided statistical consultation, analysis, technical expertise, programming, and database administration for many projects within the University of Rochester Medical Center.

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Neil E. Alexis received a Ph.D. in environmental medicine from the University of Toronto in 1997 and did post-doctoral research training at the University of North Carolina (UNC) at Chapel Hill/U.S. EPA Human Studies Facility. He is currently a professor, in the Department of Pediatrics, UNC Chapel Hill, and the director of the Applied Immunobiology Laboratory and principal investigator of the Human Sample Biorepository at the UNC Center for Environmental Medicine, Asthma and Lung Biology. His research interests focus on examining the health effects of air pollution in individuals with pre-existing airways disease and investigating the underlying pathophysiological mechanisms of airways diseases. He has focused in particular on the inflammatory and innate immune response in the airways and on the use of induced sputum as a primary sampling tool for measuring cellular, biochemical, and genetic outcomes in the airways of human subjects.

Peter Ganz received his M.D. from Harvard Medical School, completed his residency in cardiology at the Massachusetts General Hospital, and held a

cardiovascular fellowship at the Brigham and Women's Hospital, Boston, Massachusetts. He spent 25 years directing research in the Cardiac Catheterization Laboratories at the Brigham and Women's Hospital and Harvard Medical School, prior to arriving at the University of California at San Francisco in 2008. He is chief of cardiology and the director of the Center of Excellence in Vascular Research at the San Francisco General Hospital. He is the Maurice Eliaser Distinguished Professor of Medicine at the University of California, San Francisco. Ganz has been a pioneer and a leader in translational vascular research focusing on understanding key elements of human atherosclerosis, including the pathobiology of the human endothelium, the biology of vascular nitric oxide, systemic and vascular inflammatory responses, and atherosclerotic plaque instability.

Wojciech Zareba received both his M.D. and Ph.D. in cardiology from the Medical University of Lodz, Poland. Currently, he is a cardiologist and professor in the Division of Cardiology, Department of Medicine and the Heart Follow-up Program at the University of Rochester School of Medicine and Dentistry in Rochester, New York. Zareba has directed analyses of electrocardiogram (ECG) recordings for studies examining associations between markers of heart rate variability, repolarization, and other parameters and increased air pollutant concentrations. He is a cardiologist and has served as principal investigator of a number of large clinical trials testing the clinical effectiveness and safety of implantable cardiac devices, as well as several grants on risk stratification of cardiac death, clinical usefulness, and prognostic significance of ECG parameters.

OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

Rich DQ, Thurston SW, Balmes JR, Bromberg PA, Arjomandi M, Hazucha MJ, et al. In press. Does ambient ozone or other pollutants modify effects of controlled ozone exposure on pulmonary function? Ann Am Thorac Soc.

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Research Report 192, Part 2, *Multicenter Ozone Study in oldEr Subjects (MOSES): Part 2. Effects of Personal and Ambient Concentrations of Ozone and Other Pollutants on Cardiovascular and Pulmonary Function*, D. Q. Rich and M. W. Frampton et al.

INTRODUCTION

Ozone is a reactive oxidant formed, in the presence of sunlight, through complex photochemical reactions among pollutants emitted from anthropogenic and natural sources. Although ozone in the stratosphere protects the planet from harmful ultraviolet radiation, human exposure to increased levels of ozone at ground level produces adverse health effects. Ozone is one of the six criteria pollutants regulated by the U.S. Environmental Protection Agency (U.S. EPA*) under the Clean Air Act. The effects of ozone exposure on the human respiratory system are well established and include exacerbation of asthma and increases in hospital admissions and death from respiratory illnesses, such as chronic obstructive pulmonary disease (COPD) and asthma. Data from the Global Burden of Disease initiative (GBD 2019) show that ozone is globally ranked 31st in the list of risk factors contributing to deaths from all causes, mostly due to deaths from chronic respiratory diseases (in 2017, 0.84% of deaths globally were attributed to ozone). On the other hand, the effects of ozone exposure on the cardiovascular system are not as well characterized, and research in this area has produced inconsistent results. It is plausible that ozone could cause cardiovascular dysfunction by mechanisms such as systemic inflammation, oxidative stress, and alterations in autonomic balance, and these effects can lead to endothelial dysfunction, acute arterial vasoconstriction, arrhythmias, and procoagulant activity. Recent years have seen an increase in the number of deaths attributed to ozone owing to rising ozone concentrations in countries at middle levels of development with rapidly growing economies, such as China (HEI 2019). Together, these issues point to the importance of research to fill the gap in our understanding of the cardiovascular effects of ozone, particularly at near-ambient concentrations.

As described by Frampton and colleagues (2017), HEI funded the "Multicenter Ozone Study in oldEr Subjects" (MOSES) under Request for Applications (RFA) 10-1, *Cardiovascular Effects of Exposure to Low Levels of Ozone in the Presence or Absence of Other Ambient Pollutants.* The RFA solicited research on the effects of near-ambient concentrations of ozone on the human cardiovascular system to fill an important knowledge gap. To increase the number of participants and for geographical diversity, HEI selected three clinical research centers to conduct controlled exposures to ozone in participants between ages 55 and 70 years, using a common protocol, standard operating procedures, and a common plan for data analysis. Exposure concentrations were set at 70 and 120 parts per billion (ppb), similar to ambient concentrations experienced by populations around the world (HEI 2019).

The teams were led by Dr. John Balmes at the University of California–San Francisco (UCSF), Dr. Philip Bromberg at the University of North Carolina–Chapel Hill (UNC), and Dr. Mark Frampton at the University of Rochester Medical Center (URMC), New York. The outcome measures focused primarily on cardiovascular effects; in addition, indicators of pulmonary function, inflammation, and oxidative stress were of interest as secondary endpoints. HEI formed a special MOSES Oversight Committee to provide input during the development of the study protocol and the standard operating procedures. The HEI Research Committee provided input while the study was ongoing. In addition, a MOSES Data Management Board was formed to ensure data quality and participant safety during the study.

The final report for MOSES, Part 1 (MOSES 1) describes in detail the controlled human exposure study to evaluate the effects of ozone exposures in a panel of healthy volunteers, with moderate levels of exercise (Frampton et al. 2017; see also Arjomandi et al. 2018; Balmes et al 2019; Rich et al. 2018, 2020). The current report for MOSES, Part 2

Drs. David Q. Rich and Mark W. Frampton's 1-year study, "The Multicenter Ozone Study in Elderly Subjects (MOSES) 2: Impacts of personal and ambient concentrations of ozone and other pollutants on cardiovascular and pulmonary function," began in January 2017. Total expenditures were $216,402. The draft Investigators' Report from Rich, Frampton, and colleagues was received for review in July 2018. A revised report, received in February 2019, was accepted for publication in June 2019. During the review process, the HEI MOSES Review Panel and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Report and the MOSES Review Panel's Commentary. (As a coinvestigator of the MOSES report, Dr. Frampton, who was a member of the HEI Review Committee, was not involved in its evaluation by the MOSES Review Panel.)

* A list of abbreviations and other terms appears at the end of this volume.

(MOSES 2) describes additional analyses to evaluate whether the MOSES 1 results were in any way affected by normal, daily exposures to ambient air pollution as experienced by the participants in the days leading up to the clinical visits, as explained in more detail below.

SUMMARY OF MOSES 1

The three centers successfully recruited and tested 87 participants (average age 60 years, range 55 to 70 years) who each completed three exposure visits between July 2012 and April 2015. Sixty percent of participants were women, 88% were white, and 57% were lacking the GSTM1 gene, which plays a role in the detoxification of metabolites of environmental toxicants and was hypothesized to possibly enhance effects of ozone exposure. In 20 participants, 39 mild-to-moderate adverse events were recorded, mostly headache attributed to caffeine withdrawal. Some participants reported nasal congestion or fatigue. None of these symptoms were found to be related to ozone exposure. None of the participants withdrew from the study because of adverse events.

Analyses of the primary cardiovascular endpoints found no statistically significant changes following 3-hour ozone exposure at 70 or 120 ppb on autonomic nervous system function, cardiac electrical repolarization, or cardiac arrhythmia. In addition, ozone exposure did not lead to significant changes in markers of systemic inflammation and oxidative stress (C-reactive protein, interleukin-6, 8-isoprostane, and P-selectin), vascular function (blood pressure and flow-mediated dilatation of the brachial artery), or prothrombotic status (microparticle-associated tissue factor activity and monocyte–platelet conjugate count). Of the cardiovascular endpoints considered, ozone exposure was only associated with increased plasma endothelin-1 and decreased nitrotyrosine (both markers of endothelial function) after exposure to 120 ppb, but not 70 ppb, ozone.

MOSES 1 confirmed that ozone has effects on the respiratory system in these older healthy participants at these near-ambient concentrations. Moderate exercise during exposure to clean air (0 ppb) led to an increase in forced vial capacity (FVC) and forced expiratory volume in one minute (FEV_1) 15 minutes after the end of exposure compared with pre-exposure values, and those lung function markers remained statistically significantly higher 22 hours after the end of exposure. These improvements were attenuated after ozone exposure at both 70 and 120 ppb. In addition, ozone exposure at 120 ppb significantly increased the percentage of neutrophils (a marker of lung inflammation) in sputum as well as of levels of CC16 (a marker of airway epithelial cell injury) in blood 22 hours later, compared with clean air. In contrast, controlled exposure to ozone was not associated with changes in sputum concentrations of the inflammatory markers (interleukin-6, interleukin-8, and tumor necrosis factor [TNF]-α). There was no evidence of statistically significant interactions between sex, age, or GSTM1 status and the observed changes in lung function, sputum neutrophils, or plasma CC16 after ozone exposure. In summary, MOSES 1 found effects of controlled ozone exposure at 70 and 120 ppb on lung function and biomarkers of inflammation, but not on the primary cardiovascular markers of interest.

MOSES 1 noted some differences among centers in average personal exposures to ozone and nitrogen dioxide (NO_2) of participants during the three days before the exposure visits. Specifically, average personal exposures to NO_2 were lower at UNC than at UCSF or URMC, whereas they were lowest for ozone at UCSF. An overview of mean personal and ambient exposure concentrations for the three centers over the study period (2012–2015) is provided in Commentary Table 1.

There were also some differences among the study centers in ambient concentrations of air pollutants measured at central air quality monitors. For example, ambient NO_2 and carbon monoxide (CO) levels (measured at central monitoring sites and averaged over the entire study period) were higher at UCSF than at the two other centers. Those differences were further explored in MOSES 2 by including ambient exposures of the participants in the statistical analyses of the controlled ozone exposures.

GOALS OF MOSES 2

There is interest in assessing how exposure to ambient air pollutants in the days leading up to the controlled exposures might influence the results, in particular because the controlled exposure levels in this study (i.e., 70 and 120 ppb) were — by design — close to ambient ozone concentrations. Thus, the three teams measured each participant's exposure to ozone and NO_2 using a personal sampler for 72 hours before the pre-exposure visit. They also collected air quality data for ozone, particulate matter ≤ 2.5 μm in aerodynamic ($PM_{2.5}$), NO_2, sulfur dioxide (SO_2), and CO from central monitors close to each clinical center for up to 96 hours prior to the clinical visit.

After completion of MOSES 1, the team at URMC led by Drs. David Rich and Mark Frampton conducted additional data analyses to evaluate the effects of such prior exposures, presented in the current report. The investigators used the same statistical approach as in MOSES 1, but with inclusion of the prior ambient exposure data in the models. They also conducted various sensitivity analyses, for example, to look at the effects of using logarithmic transformations of some of the biomarker data. The investigators

Commentary Table 1. Personal and Ambient Concentrations of Pollutants for MOSES Participants

	Personal		Ambient				
Clinical Center	Ozone (ppb)	NO_2 (ppb)	Ozone (ppb)	NO_2 (ppb)	SO_2 (ppb)	CO (ppm)	$PM_{2.5}$ (μg/m3)
Mean Concentration and Standard Deviation[a]							
UCSF	3.70 ± 3.24	14.44 ± 10.98	22.88 ± 7.46	11.26 ± 8.33	No data	0.37 ± 0.13	8.80 ± 4.29
UNC	3.83 ± 3.65	4.00 ± 5.85	28.64 ± 8.44	6.07 ± 2.97	0.29 ± 0.46	0.19 ± 0.04	8.09 ± 3.05
URMC	3.93 ± 4.65	10.04 ± 9.77	26.48 ± 7.02	6.45 ± 3.09	1.02 ± 0.55	0.20 ± 0.05	6.50 ± 2.33
Maximum Concentration[b]							
UCSF	13.30	71.35	46.90	45.30	No data	0.86	37.92
UNC	17.34	29.78	64.00	35.20	7.30	0.52	27.40
URMC	20.03	72.36	74.00	20.50	10.12	0.49	31.30

Abbreviations: CO = carbon monoxide; NO_2 = nitrogen dioxide; $PM_{2.5}$ = particulate matter ≤ 2.5 μm in aerodynamic diameter; SO_2 = sulfur dioxide; UCSF = University of California San Francisco; UNC = University of North Carolina; URMC = University of Rochester Medical Center.

[a] Mean concentration and standard deviation for participants' personal exposure measurements of ozone and NO_2 during 72 hours prior to the visit to the clinic, or mean concentration and standard deviation of five ambient pollutants measured at a central monitor closest to the clinic, calculated over the entire study period (2012–2015).

[b] Maximum concentration for participants' personal exposure measurements of ozone and NO_2 during 72 hours prior to the visit to the clinic, or maximum concentration of five ambient pollutants calculated over the study period during any of the lag times (0 to 96 hours) prior to the visit to the clinic. (See Investigators' Report Tables 3 and 4.)

pursued four specific aims to evaluate whether there was confounding or effect modification of the results of MOSES 1 by pre-baseline exposures to ambient air pollution, whether prior exposures led to differences in baseline values of the biological markers, and whether prior pollutant exposures were associated with pre- to post-ozone changes in biomarkers, adjusted for the controlled ozone exposures.

This Commentary provides the HEI MOSES Review Panel's evaluation of Part 2 of the MOSES study. It is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study and by placing the Investigators' Report into scientific and regulatory perspective.

SCIENTIFIC AND REGULATORY BACKGROUND

OZONE CHEMISTRY AND AMBIENT CONCENTRATIONS

Ozone is not emitted directly from combustion sources but is formed by chemical reactions of precursors such as nitrogen oxides (NO_x) and volatile organic compounds (VOCs) in the presence of sunlight. Sources of ozone precursors are both manmade (i.e., anthropogenic) and natural. Sources of NO_x include motor vehicles, power plants, and wildfires. VOC sources include motor vehicles, paints and solvents, wildfires, and vegetation (e.g., plants emit VOCs such as pinene and isoprene). Modeling of ozone concentrations in the atmosphere needs to take into account the emissions of those precursors, atmospheric chemistry, meteorology, and transport.

People across the globe are exposed to varying amounts of ozone in the air they breathe. Background levels of ozone, mainly of natural origin, are estimated to be in the range of 20–35 ppb and may be increased by intercontinental transport of anthropogenic pollution (U.S. EPA 2013, 2019). For regulatory purposes, ozone concentrations are generally calculated as seasonal averages, primarily during the warmer months when ozone levels are highest due to increased solar radiation. Depending on location, the warm season during which states are required to report ozone concentrations varies from March through November (for southern states) to June through September

(for northern states). In the United States, the median ozone concentration reported for 2009 was 40 ppb (maximum value, 188 ppb; 8-hr daily maximum) across 1,141 nationwide monitors that measured ozone during the warm season. Depending on the level of urbanization and various local factors, many counties exceed an annual average of 60 ppb (8-hr daily maximum) (U.S. EPA 2013, 2019).

HEI's State of Global Air website presents ozone levels across the world for the past decade; note that the values are calculated as average concentrations (of 8-hr daily maximum values) during the warm season (which varies by location) and as population-weighted averages — meaning that concentrations in urban areas were given more weight than concentrations in rural areas where fewer people live. The numbers have been revised in the latest State of Global Air (HEI 2019), now incorporating a more extensive database of ground-level ozone measurements. Seasonal population-weighted concentrations for 2017 ranged from about 20 to 30 ppb, mostly in small island nations, to 60 to 70 ppb in Asia and the Middle East. Global average concentrations have been stable at about 57 ppb between 1990 and 2017 (HEI 2019). The more developed regions, such as North America, continue to experience high ozone exposures, despite extensive and successful air quality control for ozone-related emissions. In countries with moderate levels of development and rapidly growing economies, such as China, population-weighted ozone concentrations have been increasing slowly but steadily (HEI 2019). With rising global temperatures, average and peak concentrations of ozone are expected to increase, with potentially important consequences for human health (Atkinson et al. 2016; Chang et al. 2010; Fann et al. 2015; Karlsson et al. 2017). Thus, large sections of the global population continue to be exposed to unhealthy levels of ozone. In the latest Global Burden of Disease analysis, ozone was ranked as the 31st highest risk factor for deaths globally, mostly due to deaths from COPD (GBD 2019).

HEALTH EFFECTS OF OZONE

Ozone is known to have both short-term and long-term effects on human health, with the strongest evidence for respiratory effects and some evidence for effects on the cardiovascular and other organ systems at current ambient concentrations. In determining whether ozone exposure is causally related to certain health outcomes, scientists consider evidence from human epidemiology studies and controlled human exposure studies; they also consider results from animal research to support the findings in humans and strengthen knowledge about mechanistic pathways.

The evidence has been described in detail in the recent *Integrated Science Assessment for Ozone and Related Photochemical Oxidants* by the U.S. EPA (2019). Here, we focus on key additional evidence that has become available since the previous Integrated Science Assessment was conducted in 2013.

Respiratory Effects

With rising global temperatures, average and peak concentrations of ozone are expected to increase, with potentially important consequences for human health (Atkinson et al. 2016; Chang et al. 2010; Fann et al. 2015; Karlsson et al. 2017). It is well known that ozone adversely affects the lungs. After inhalation, ozone reacts with constituents of the respiratory tract lining fluid to generate reactive oxygen species that can overwhelm antioxidant defenses and cause local oxidative stress in the respiratory tract. Ozone's high reactivity makes it unlikely that it penetrates far beyond the fluid that lines the lung's epithelial cell layer. Its harmful effects are thought to be mediated by products of its reactions with constituents of the lining fluid and the epithelial cell membrane (Pryor 1992) that may travel beyond the lung to produce effects elsewhere in the body.

Short-term effects of ozone exposure include shortness of breath, exacerbation of asthma symptoms and greater medication use, and increases in respiratory-related hospital admissions and emergency department visits related to asthma. Some evidence links short-term ozone exposure to mortality (Olstrup et al. 2019). Time-series epidemiological studies have shown associations of short-term exposure to ozone and daily deaths (Ito et al. 2005; Peng et al. 2013; Olstrup et al. 2019). Long-term exposure to ozone has been associated with increased mortality among Medicare enrollees who had previously been hospitalized because of COPD (Zanobetti and Schwartz 2006) and with increased risk of acute respiratory distress syndrome in older adults (Rhee et al. 2019). Evidence is mounting for associations between long-term exposure to ozone and deaths from both respiratory and cardiovascular causes in cohort studies (Atkinson et al. 2016; Balmes 2019; Lim et al. 2019; Paulin et al. 2019; Turner et al. 2016).

Cardiovascular Effects

Evidence for effects of ozone on cardiovascular outcomes has been inconsistent, but recent studies have added to the evidence base. It remains difficult to disentangle the cardiovascular effects of ozone from those of other air pollutants, especially particulate matter (PM), which has been shown to have strong associations with cardiovascular deaths and illnesses. However, some recent studies suggest that long-term ozone exposure may be related to cardiovascular deaths in the general population

(Cakmak et al. 2016; Lim et al. 2019; Turner et al. 2016) and in older individuals who had a history of congestive heart failure or myocardial infarction (Zanobetti and Schwartz 2006). In a large cohort of African American women, chronic exposure to higher ozone levels was associated with higher incidence rates of hypertension (Coogan et al. 2017) and type 2 diabetes (Jerrett et al. 2017). Other studies have shown increased rate of carotid artery wall thickness and risk of new plaque formation (Wang et al. 2019), as well as increased rates of hospital admissions of Medicare patients for stroke, COPD, pneumonia, myocardial infarction, lung cancer, and heart failure associated with long-term exposure to ozone (Danesh Yazdi et al. 2019).

Epidemiological studies have found positive associations between short-term ozone exposure and deaths from all causes, in particular during the warm season (when ozone levels are typically higher than in the cold season) and have found that these associations are independent of co-exposure to PM (U.S. EPA 2013, 2019). A 2016 review of panel studies concluded that effects of short-term ozone exposure on heart rate variability remained inconclusive (Buteau and Goldberg 2016). Since then, short-term exposure to ozone was found to be associated with an elevated risk of out-of-hospital cardiac arrest (Raza et al. 2019) and with various electrocardiogram (ECG) measures in patients undergoing cardiac catheterization (Zhang et al. 2018).

Controlled Exposure Studies in Volunteers

Some of the most convincing evidence of the short-term effects of acute ozone exposure has been provided by human controlled-exposure studies, primarily conducted in healthy young adults. Short-term controlled exposure to low concentrations of ozone — close to the current U.S. National Ambient Air Quality Standard (NAAQS) of 70 ppb — during intermittent exercise decreases lung function and increases airway hyperreactivity and airway inflammatory responses (U.S. EPA 2019). The mechanisms by which ozone induces acute effects in humans were reviewed by Bromberg (2016).

The strongest evidence for short-term effects of ozone exposure on cardiovascular outcomes has been provided by animal studies, with more limited evidence from panel studies in volunteers. Short-term ozone studies in animals have reported changes in heart rate, heart rate variability, arrhythmias, and vascular reactivity; some of these effects overlap with those observed after long-term ozone exposure (U.S. EPA 2013, 2019). Not all evidence is straightforward, however; for example, both increases and decreases in heart rate have been observed. Possible pathways include systemic oxidative stress and changes in the autonomic nervous system that are triggered by inflammation in the lung. Some recent studies have shown effects at concentrations as low as 60 ppb (Adams 2006).

Summary of Evidence

In its 2019 *Integrated Science Assessment for Ozone and Related Photochemical Oxidants*, the U.S. EPA summarized the health effects of ozone exposure as follows:

"Recent evidence continues to support ozone-induced effects on the respiratory system. In addition, recent evidence indicates ozone-induced metabolic effects…. There is also some evidence that ozone exposure can affect the cardiovascular and nervous systems, reproduction and development, and mortality, although there are more uncertainties associated with interpretation of the evidence for these effects."

Based on the overall evidence on health effects of ozone summarized in the 2013 Integrated Science Assessment, the 8-hour NAAQS for ozone was set at 70 ppb in 2015. The ozone standard is currently in its 5-year review cycle following completion of the most recent draft Integrated Science Assessment (U.S. EPA 2019).

At the time the MOSES study started, there was a clear need for a study in volunteers that would investigate the potential cardiovascular effects of short-term ozone exposures to near-ambient concentrations. The following section summarizes the study's approach and key results and is followed by an evaluation of the study's strengths and limitations as assessed by the HEI MOSES Review Panel in its independent review of the study. It provides a summary of MOSES 1 results and then focuses in more detail on MOSES 2.

SUMMARY OF THE STUDY

SPECIFIC AIMS

MOSES 1 evaluated the effects of short-term controlled exposure to 70 and 120 ppb ozone on the cardiovascular and respiratory systems in participants aged 55 to 70 years. MOSES 2 investigated whether prior exposures to ambient air pollutants (ozone, NO_2, $PM_{2.5}$, SO_2, and CO) affected the outcomes of the controlled exposures. The investigators evaluated the effects of personal exposures to ozone and NO_2 using data collected from a personal sampler for 3 days (72 hours) before the visit to the clinic. They also evaluated the effects of exposure to ambient concentrations for up to 4 days (96 hours) prior to the visit to the clinic, using data collected from central monitors closest to each clinical center.

MOSES 2 had four specific aims. They were to assess:

1. Whether any changes in biomarkers measured before and after the controlled ozone exposures were *confounded* by prior exposures to ambient air pollutants. That is, whether inclusion of ambient pollutant exposures in the analytical models altered the estimate of changes in biomarkers observed after controlled ozone exposures.
2. Whether there was *effect modification* by the prior exposures of any changes in biomarkers measured before and after the controlled ozone exposures. That is, whether experimental ozone effects (on cardiovascular biomarkers in particular) could only be seen when prior ambient exposures were low, indicating that high prior exposures may have masked the experimental effects, or only when they were high, indicating that the experimental exposures enhanced already existing effects from daily ambient pollutant exposures.
3. Whether prior pollutant exposures were associated with differences in *baseline values* of the biological markers measured before the start of the three experimental ozone exposures at 0, 70, and 120 ppb.
4. Whether prior pollutant exposures had *independent effects* on changes in biomarkers, adjusted for controlled ozone concentration.

STUDY DESIGN AND METHODS

Controlled Ozone Exposures in MOSES 1

As detailed in the MOSES 1 report (Frampton et al. 2017), 87 participants, who were nonsmokers and had normal lung function and normal ECGs, completed three exposure sessions (randomized at 0, 70, and 120 ppb ozone) with a minimum of 2 weeks between exposure sessions. Exposures lasted 3 hours, during which the participants exercised on a stationary bicycle or treadmill, alternating 15 minutes of exercise with 15 minutes of rest. Each exposure session included 3 days of visits to the clinical center: on the pre-exposure day, the exposure day, and the post-exposure day. Participants stayed in a nonsmoking hotel room the night before the exposure day to minimize variability in exposure to ambient air pollutants.

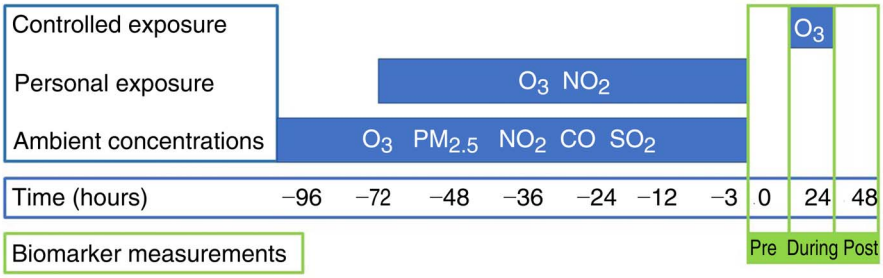
The three investigator teams measured a large suite of endpoints before, during, and up to 22 hours after exposure, including changes in heart rate, heart rate dynamics, blood pressure, pulmonary function, and markers of endothelial function, thrombosis, lung injury, and both systemic and lung inflammation (see Investigators' Report Table 1**)**. They prespecified a key group of cardiovascular endpoints as primary endpoints and considered all other endpoints as secondary. Most outcomes were assessed at designated central core laboratories that handled samples, electrocardiographic recordings, or ultrasound images from all three clinical centers in order to standardize the outcome assessments, and all data were deposited and analyzed at a central data management center. Study participants were also genotyped for GSTM1 variants. The three clinical centers obtained appropriate approvals from their respective Institutional Review Boards.

Prior Exposures to Ambient Air Pollutants

Personal exposure to ozone and NO_2 was measured using an Ogawa personal exposure sampler (PES) for 72 hours preceding the pre-exposure visit. After the samplers were returned to the clinical centers by the participants, the samples were refrigerated together with blank samples until they were shipped to RTI International for analysis. Participants were also asked to fill out an activity diary during that time. In addition, hourly air quality (ozone, $PM_{2.5}$, NO_2, CO, and SO_2) and meteorological (temperature and relative humidity) data were collected from central monitoring stations closest to each of the three centers for the entire study period (2012–2015). All data were checked for outliers. Some concentrations were less than zero. The investigators did not correct those values because they did not want to alter the distribution, and thus the interquartile range was used to scale effect estimates. An overview of the timing of the exposure and biomarker measurements is provided in Commentary Figure 1.

Statistical Analyses

A statistical analysis plan for MOSES 1 was developed with input from investigators at the three clinical centers and the HEI MOSES Oversight Committee. All statistical analyses for MOSES 1 were done at the New England Research Institute. First, data were assessed for outliers and verified at the source if they looked suspicious, and corrected if reliable information was available. Results were then calculated as the difference between pre-exposure and post-exposure values and assessed for normality. For outcomes that were not normally distributed, a natural log transformation was performed. Effects of ozone exposure on primary and secondary health outcomes were analyzed using mixed-effects linear models accounting for repeated measures (at multiple time points). In MOSES 1, the investigators tested three interaction models: ozone by sex, by age, or by GSTM1 status; numerous subgroup analyses were also performed. The statistical significance threshold was set at $P < 0.01$ in light of multiple comparisons.



Commentary Figure 1. Overview of personal, ambient, and controlled exposures to ozone (O_3) and other pollutants in the MOSES project. Green boxes indicate three days of clinic visits for biomarker measurements before, during, and after a 3-hour controlled exposure to ozone.

For MOSES 2, the investigators used the same statistical models as in MOSES 1, but with inclusion of prior pollutant exposure data. For Aim 1 (confounding), the investigators analyzed all biomarker data (i.e., the difference between pre- and post-ozone exposure) using linear effect models with the addition of air pollutant data, temperature, and relative humidity. The model was run multiple times, with the inclusion of personal exposure for 72 hours to ozone or NO_2, or ambient exposure to ozone, $PM_{2.5}$, NO_2, CO, or SO_2 for various time lags (0, 3, 12, 24, 48, 72, or 96 hours prior to the clinical visit). Thus, all MOSES 1 biomarker data were reanalyzed using a total of 37 statistical models.

For Aim 2 (effect modification), the investigators analyzed all primary (cardiovascular) biomarker data using the same approach as that described for Aim 1, but with the pollutant concentrations divided into tertiles. They then included the interaction between the pollutant tertiles and the controlled ozone exposure in the model to determine whether high or low prior exposures to ambient pollutants affected the change in biomarkers following the controlled ozone exposures. The investigators had two a priori hypotheses: Controlled ozone effects on biomarkers would be greater if ambient exposures were low, because high ambient exposures would make it more difficult to see any effects of the experimental ozone exposures (Hypothesis #1). Alternatively, controlled ozone effects on biomarkers would be greater if ambient exposures were high, requiring a "priming" effect of the ambient exposures in order to see an effect of the controlled ozone exposures (Hypothesis #2). After analyzing the primary biomarkers using the exposure tertiles, the investigators analyzed the corresponding secondary biomarkers as well if results for primary outcomes showed consistency with either of the hypotheses.

For Aim 3 (baseline values), the investigators used a linear mixed-effects model with the outcome defined as the three baseline observations for each participant, that is, the pre-exposure measures for all biomarkers that preceded the controlled exposures to 0, 70, and 120 ppb ozone. They included personal (72 hours) and ambient exposures (all lags) as well as temperature and relative humidity as described for Aim 1.

In addition, the investigators conducted several sensitivity analyses for Aim 3. First, they evaluated the effect of log-transforming the data. Second, they evaluated whether inclusion of relative humidity in the models mattered. Leaving out relative humidity, which had a large number of missing values, increased the sample size because it reduced the number of missing data for ambient ozone, $PM_{2.5}$, CO, and NO_2.

For Aim 4 (independent effects of ambient pollutants, adjusted for controlled ozone concentration), the investigators used the same analysis of primary biomarker data as described for Aim 1; some sensitivity analyses included two pollutants in the same model. They also analyzed each biomarker–pollutant combination to determine at what lag period the largest effect was observed. Those lags were then used in the analyses of Aim 2.

SUMMARY OF RESULTS

Commentary Table 2 provides an overview of the MOSES 2 analyses. Overall, the investigators looked for consistency in the results for primary and secondary biomarkers, whether changes were in the expected direction, whether there were consistent dose–response relationships, and whether the results were biologically plausible.

Commentary Table 2. Overview of MOSES 2 Analyses and Findings

Specific Aim	Biomarkers Analyzed[a]	Pollutants and Time Lags Analyzed[b]	Statistical Models	Main Findings
1: Confounding	All	All pollutants and lags	Linear mixed effects model, with inclusion of each pollutant and lag time	No evidence of confounding by prior exposures
2: Effect modification	Primary and selected secondary biomarkers	All pollutants at one selected lag[c]	Same as Aim 1, with pollutant concentrations by tertiles, and an interaction term	No evidence of effect modification by pollutant tertile, except for lung function changes, which were enhanced by prior higher NO_2 or CO exposure.
3: Effect on baseline values	All	All pollutants and lags	Same as Aim 1, but using only the 3 pre-exposure baseline values	Possible associations of ambient ozone with baseline HRV; possible association of ambient $PM_{2.5}$, CO, and NO_2 with baseline C-reactive protein and lung function [d]
4: Independent effects	Primary and selected secondary markers	All pollutants and lags	Same as Aim 1; some analyses included two pollutants in the model	Possible ambient ozone associations with high-frequency HRV were independent of ambient $PM_{2.5}$, CO, and NO_2

Abbreviations: CO = carbon monoxide; HRV = heart rate variability; NO_2 = nitrogen dioxide; PES = personal exposure sampler; $PM_{2.5}$ = particulate matter ≤ 2.5 µm in aerodynamic diameter.

[a] The investigators analyzed a broad set of biomarkers, grouped as follows: Autonomic balance of the heart (heart rate variability, repolarization, and arrhythmia); systemic inflammation, oxidative stress, and vascular function; and prothrombotic vascular state. For each of these groups, certain markers were a priori selected as primary markers of interest and the remaining markers were secondary. Two additional groups of markers were sputum and plasma markers of airway inflammation and lung injury, and lung function, which were designated as secondary markers.

[b] Personal exposure concentrations: PES ozone and PES NO_2 (both measured over 72 hours prior to the visit to the clinic); as well as ambient concentrations of ozone, NO_2, CO, SO_2, and $PM_{2.5}$ (estimated at lags 0, 3, 12, 24, 48, 72, and 96 hours prior to the visit). Pollutant concentrations were divided into tertiles (low, medium, and high).

[c] Analyses were conducted at the lag with the largest observed effect, as analyzed under Aim 4.

[d] The investigators noted a "recovery" effect, where participants showed a change in baseline values while breathing clean air during the pre-exposure period.

In MOSES 2, the investigators found that prior exposures did not confound the effects of controlled ozone exposures on primary and secondary cardiovascular or pulmonary endpoints as reported in MOSES 1 (Aim 1, confounding). The endpoints that changed significantly in MOSES 1 (lung function and markers of inflammation) remained statistically significant after further adjustment for prior exposures. Some minor changes were observed, such as a diminished effect of controlled ozone exposure on FEV_1 upon further adjustment for ambient NO_2 during one hour prior to the pre-exposure visit. However, this was only one of 37 analyses performed with all ambient pollutants at all lag hours. Similarly, the estimates of controlled exposure to ozone on cardiovascular outcomes and markers of oxidative stress and coagulation were consistent even after adjustment for prior exposures.

Aim 2 analyses using low, medium, and high tertiles of personal and ambient pollutant exposures found no effect modification or inconsistent evidence for most primary cardiovascular biomarkers. For the lung function measures

FEV_1 and FVC only, some evidence was reported for effect modification by ambient concentrations of NO_2 (72 hours prior) and CO (3 hours prior) and of personal exposure to NO_2 (72 hours prior), when concentrations of those pollutants were in the high tertile (often with a trend in the same direction for the medium tertile). Examples are provided in Commentary Figure 2. The evidence supports Hypothesis #2, indicating that controlled ozone exposure enhanced an already existing effect of daily ambient exposure to CO and NO_2 (but not ozone or other pollutants) on lung function, particularly at 120 ppb.

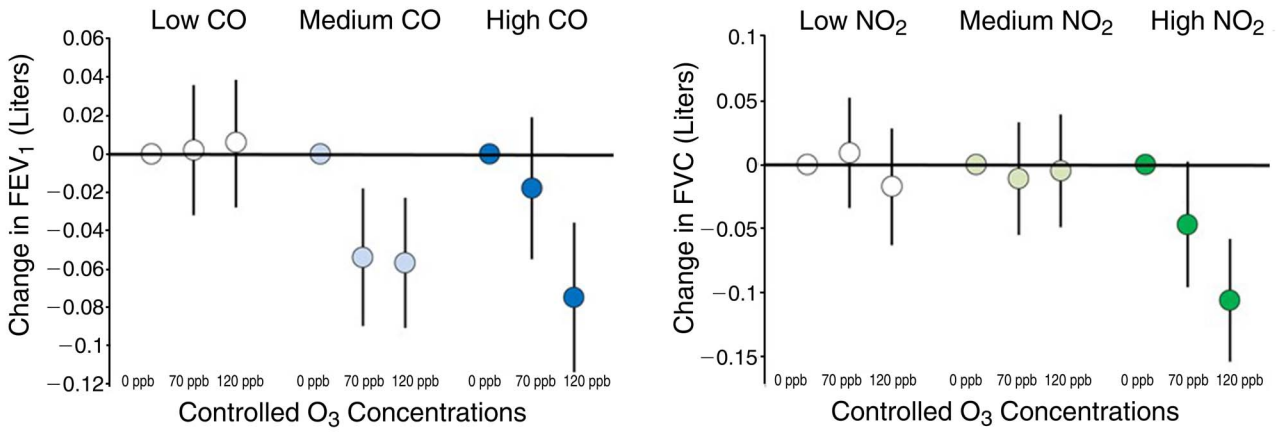
The investigators analyzed Aims 3 and 4 together, because they found some connection between differences in pre-exposure baseline values and ambient pollutant interactions associated with changes in biomarkers after controlled ozone exposure. They noted that ambient exposure to ozone decreased several baseline (pre-exposure) heart rate variability measures, a potentially adverse effect. An example is provided in Commentary Figure 3, showing baseline changes in high-frequency power. However, ambient ozone was associated with increases in frequency-domain heart rate variability following experimental visits. The investigators interpreted this as a "recovery" or rebound of the observed decreases in baseline heart rate variability.

Similar patterns were observed for lung function measures FEV_1 and FVC, with decreased baseline values associated with increased ambient exposure to $PM_{2.5}$, NO_2, and CO, independent of ambient ozone exposures. Again, changes were in the opposite direction, showing a "recovery" of those measures during the experimental visits. There were no effects of ambient pollutants on changes in the other biomarkers, or the effects were inconsistent. There appeared to be no effect on the baseline values of personal exposures of O_3 and NO_2.

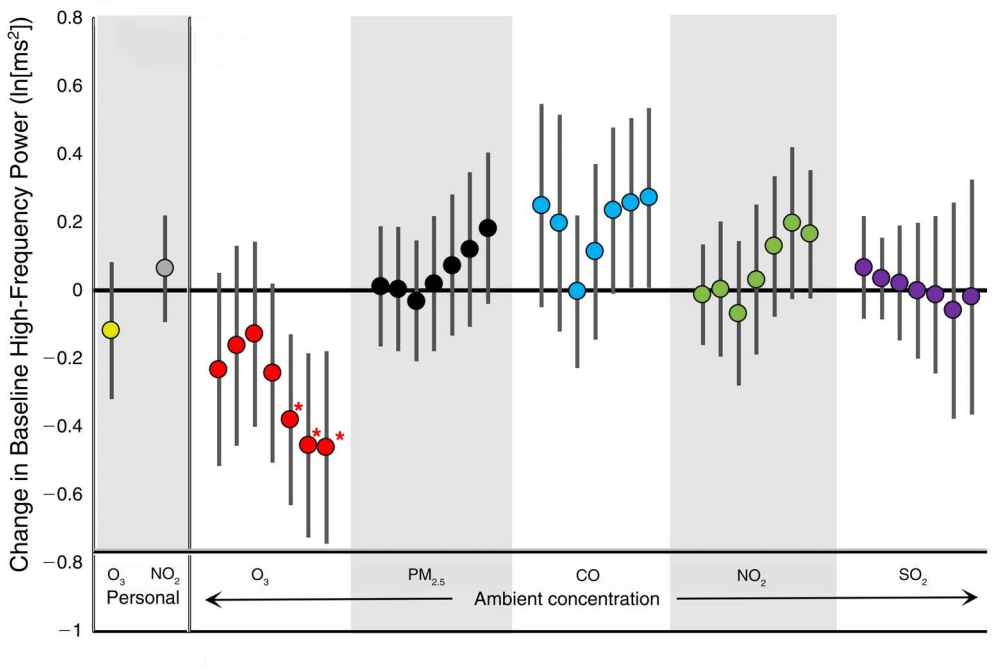
EVALUATION BY THE HEI MOSES REVIEW PANEL

In its independent review of the study, the HEI MOSES Review Panel of the HEI Review Committee commended the investigators for a well-designed and well-executed follow-on study to MOSES 1. In addition to the key analysis done for Aim 1 (possible confounding of MOSES 1 results), they evaluated various other research questions to understand how daily ambient pollutant exposures may have affected baseline levels of biomarkers and whether the pollutants interacted with each other.

A key strength of MOSES was the crossover design with controlled exposures at three concentrations (0, 70, and 120 ppb) with the participants and most laboratory personnel unaware of the exposure concentrations. In addition, the number of participants in the MOSES study was considerably larger than in previous human exposure



Commentary Figure 2. Influence of ambient concentrations during preceding days on changes in lung function after controlled ozone exposure to 70 or 120 ppb ozone. Ambient pollutant concentrations up to 72 hours prior to ozone exposure were divided into tertiles. Changes were seen only when ambient concentrations were in the middle or high tertiles. Left: FEV_1 and ambient carbon monoxide. Right: FVC and ambient nitrogen dioxide.



Commentary Figure 3. Baseline high frequency power, a measure of heart rate variability, showing association with prior ambient ozone concentrations (red symbols) but not with other pollutants. Changes in high frequency are expressed per interquartile range of pollutant concentration. Results are shown for 37 statistical analyses that include either personal exposure to ozone (yellow) or NO_2 (grey) measured over 72 hours, or mean ambient pollutant concentrations (various colors) over 0, 3, 12, 24, 48, 72, and 96 hours prior to the baseline measurement. *$P < 0.01$.

studies conducted to date. The Panel thought the study had sufficient statistical power to detect changes of a relevant magnitude in the primary outcomes.

The MOSES 2 report presents a very large number of informative additional analyses with the goal to determine whether the MOSES 1 results may in any way have been affected by participants' daily exposures to ambient ozone or other air pollutants, such as $PM_{2.5}$ and NO_2, prior to the controlled exposure. This question was particularly relevant because the controlled ozone exposures in MOSES 1 were — by design — close to ambient ozone concentrations. The Panel commended the investigators for conducting these analyses and thought the statistical analyses and interpretation of results had been done appropriately. The Panel agreed with the report's main conclusion that the MOSES 1 results were not confounded by the participants' immediate prior exposures to air pollutants.

In summary, MOSES 1 showed that 3-hour ozone exposure at 70 or 120 ppb did not lead to statistically significant changes in cardiovascular endpoints in this healthy group of older participants undergoing moderate exercise. However, exposure to ozone led to moderate adverse changes in lung function measures (FEV_1 and FVC) and in two inflammatory markers (CC16 and lung neutrophils). The fact that there were concordant findings for respiratory outcomes, and that those effects were larger at 120 ppb than at 70 ppb ozone, strengthens the overall conclusion that there were respiratory, but no cardiovascular, effects observed in this group of participants. MOSES 2 has provided additional confidence in these results, supporting the conclusion that adverse lung effects (but not cardiovascular effects) were observed at ozone concentrations near the current U.S. 8-hour NAAQS of 70 ppb.

In the next sections, we discuss strengths and limitations of different aspects of MOSES 2.

CONTROLLED OZONE EXPOSURES

As was discussed in MOSES 1, the study included a one-night hotel stay before the exposure session to minimize variation in exposure to ambient air pollutants for an individual over time and across individuals. The investigators

stated that a longer stay would have been preferable but would be inconvenient to participants and add to the costs. At the time, the Review Panel noted that a one-night hotel stay may not have sufficiently eliminated the effects of prior exposure to background concentrations of ozone and other pollutants, because acute effects of air pollution have been shown to occur with a lag time of up to three days (Schwartz 2000). The current analyses shed some light on this issue. First, when associations were observed with ambient exposures, they occurred only at longer lags (48 to 96 hours). Second, the investigators noted that ambient exposures were associated with changes in baseline values of certain biomarkers, but that they observed a "rebound" effect during and after the experimental exposures, suggesting that there was a measurable "beneficial" effect of breathing relatively cleaner air for one day. This is reassuring and suggests that future researchers may consider providing clean air environments for at least one day before conducting controlled exposure experiments.

PERSONAL AND AMBIENT EXPOSURES

The investigators collected 72-hour personal samples of ozone and NO_2 using Ogawa samplers. These samplers are standard and widely used, but there was no record of whether participants complied with the instructions for using the personal sampler. Personal ozone exposures were on average close to 4 ppb, with a maximum concentration of about 20 ppb (Commentary Table 1). These values were considerably lower than the ambient concentrations measured at the central monitors, which were 23 to 29 ppb on average, with a maximum of about 74 ppb. This is most likely the result of quenching of ozone in the indoor environments where most people spend the majority of their time. In contrast, the personal NO_2 concentrations were somewhat higher than the ambient NO_2 concentrations (personal: 4 to 14 ppb, maximum 72 ppb; ambient: 6 to 11 ppb, maximum 45 ppb), which may suggest the presence of indoor sources, such as gas stoves. The investigators stated that any uncertainty in personal exposure data due to non-compliance by participants would have resulted in minimal confounding or biased results toward the null.

The investigators estimated participants' ambient exposures by using concentrations at central monitors closest to the clinical centers. Its limitations notwithstanding, this is a commonly used approach — particularly in epidemiological studies — where residential address is often not available. In MOSES, the investigators had access to residential addresses so it may have been better if more fine-grained exposure assessment had been incorporated, to provide more personalized ambient exposure values.

There were some differences in ambient pollutant concentrations among the three clinical centers, with the highest concentrations of ozone at UNC, of SO_2 at URMC, and of NO_2, CO, and $PM_{2.5}$ at UCSF. As a result, participants from UCSF dominated the high concentration tertiles for NO_2 and CO in the Aim 2 analyses (effect modification). It is possible that other differences between the participant groups at UCSF and the other centers contributed to the results, but it is not possible to know whether that was the case. Readers should be aware that the analysis of ambient exposure tertiles disrupts the within-participant linkage in the crossover study design, because many participants will experience different ambient concentrations in one treatment level (e.g., 0 ppb ozone) than in another (e.g., 120 ppb ozone).

Both the personal and ambient exposure data contained negative values. The investigators did not correct for them, because they argued that having the original distribution of exposure values was important to estimate interquartile ranges of the pollutant concentrations. Leaving the negative values in the analyses is one of several possible approaches to handling this issue. A sensitivity analysis to determine whether not correcting for negative values may have affected the reported results would have been useful.

The investigators conducted a sensitivity analysis to evaluate the effect of relative humidity, keeping data for participants that had missing values for relative humidity because it ensured a larger data sample and thus more statistical power. However, they did not find a difference in outcomes by adjusting for relative humidity.

STATISTICAL APPROACH

A key feature of MOSES 1 was that exposures were randomized in a crossover protocol, such that there was balance across the three exposure levels. This design was a major strength of the study. However, with regard to the statistical analysis, the Review Panel was concerned about the potential implications, primarily on confounding but perhaps in other areas as well, of using an interaction term between potential effect modifiers and randomized treatment in Aim 2 analyses. By using the interaction term, the analysis no longer compares outcomes within each person, because an individual's study treatments (the controlled ozone exposure concentrations) may be in different strata of prior ambient or personal concentrations. This may have important implications for residual confounding given that the strength of the original crossover design no longer applies. In other words, the randomization works (on average) at the level of the entire study population, but not necessarily within subgroups defined by pre-randomization exposures, as used in these latter analyses.

The Panel also expressed some concern about multiple testing because the investigators conducted many statistical analyses, potentially yielding false positive associations. For example, they analyzed the influence on a particular biomarker of prior exposure to each personal or ambient pollutant at various lag times for a total of 37 analyses per biomarker. The Panel appreciated that the investigators used a *P*-value of less than 0.01 to determine statistical significance and that they focused on consistency of results rather than statistical significance in light of multiple testing; though reasonable, there is no way of knowing whether these precautions were sufficient.

CONFOUNDING AND EFFECT MODIFICATION

The investigators found no convincing evidence that there was confounding of the MOSES 1 results by prior exposure to ambient air pollutants (Aim 1). However, they found some evidence of effect modification of lung function measures in support of their second hypothesis: pre- to post-exposure changes were seen when ambient pollutant concentrations were high, suggesting that the controlled ozone exposure may have enhanced an already existing effect of ambient pollution (Aim 2). There was inconsistent evidence for prior ambient pollutant exposure effects on heart rate variability and no evidence of effect modification for any of the cardiovascular biomarkers was observed. The Panel thought the analyses were well done and they provided further confidence in the results of MOSES 1.

CHANGES IN BIOMARKER VALUES

The Panel thought the analyses of prior ambient pollutant exposures on baseline levels of the cardiovascular biomarkers (Aim 3) were interesting and consistent with current knowledge. Ambient ozone was associated with changes in baseline heart rate variability, independent of other pollutants. This aim was purely observational, however, and potentially subject to confounding in a much more substantial way than the controlled exposures in MOSES 1. The Panel found that the analyses for Aim 4 (association of prior ambient exposures with pre- to post-ozone exposure biomarker changes, adjusting for controlled ozone concentration), which the investigators interpreted together with the analyses for Aim 3, were difficult to interpret.

In MOSES 1, changes were observed in only two of the many cardiovascular endpoints: an increase in endothelin-1 and a decrease in nitrotyrosine following 3-hour exposures to 120 ppb ozone. Neither of these endpoints was prespecified as a primary outcome. The nitrotyrosine changes were in the opposite direction of what would be hypothesized for an adverse effect and remain unexplained. There were no changes in markers of systemic inflammation, lending confidence to lack of effect on cardiovascular results with 3-hour controlled ozone exposures at near-ambient levels. Whereas MOSES 1 found that 120 ppb ozone was associated with lower nitrotyrosine concentrations, MOSES 2 found that higher personal and ambient NO_2 exposures at short lag times were associated with lower baseline values of nitrotyrosine (Aim 3). Thus, levels of oxidative biomarkers such as nitrotyrosine could potentially reflect not only prooxidative effects induced by exposure to ozone or NO_2, but also antioxidant homeostatic responses, and could be the result of a balance between both mechanisms.

MOSES 1 did not find any effects on heart rate variability outcomes after controlled ozone exposure. MOSES 2 did find some associations of prior exposure to ozone, NO_2, and CO with changes in baseline heart rate variability. Analysis of heart rate variability time-domain parameters (e.g., root mean square of successive differences in normal-to-normal sinus beat intervals [RMSSD] and standard deviation of normal-to-normal sinus beat intervals [SDNN]) indicated that decreases in heart rate variability were associated with ozone exposures at longer lags (more than 48 hours); however, such changes would be expected to occur within hours of exposure. On the other hand, associations of prior exposures with frequency-domain parameters (high and low frequency power) in Aim 4 analyses were in the same direction. The high frequency power (HF) component usually reflects vagal activity, while interpretation of the low frequency power (LF) component is more controversial, considered by some as a marker of sympathetic modulation but by others as a parameter that includes both sympathetic and vagal influences (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996). Because sympathetic and parasympathetic tones usually go in opposite directions, effects on the LF and HF components are expected to go in opposite directions as well. However, in MOSES 2 changes in HF and LF occurred in the same direction instead. Therefore, the Review Panel asked the investigators to also analyze the LF-to-HF ratio, which may reflect a balance of sympathetic and vagal activities of the heart. The investigators reported no associations of prior exposures with the LF-to-HF ratio. The Panel believes that this result, together with the aforementioned long lags in associations with time-domain heart rate variability parameters, decreases confidence in the interpretation that prior exposure to ambient ozone may have affected heart rate variability.

The pulmonary changes reported with ozone exposure in the current study confirm and expand earlier findings from controlled human exposure studies. Specifically, previous studies have shown changes in pulmonary function and neutrophils after short-term (4 to 6.5 hours) exposure to low to medium (60 to 220 ppb) concentrations of ozone (Alexis et al. 2010; Balmes et al. 1996; Kim et al. 2011; Torres et al. 1997). The fact that MOSES 1 and MOSES 2 did not find cardiovascular effects of short-term ozone exposure is in contrast with some earlier studies in volunteers that reported changes in heart rate variability and a range of inflammatory and vascular biomarkers after exposure to 114 to 300 ppb ozone for 2 hours (Devlin et al. 2012; Fakhri et al. 2009), although some other studies also failed to detect effects on blood pressure after exposure to 114 to 300 ppb ozone for 2 or 3 hours (Fakhri et al. 2009; Gong et al. 1998; Ramanathan et al. 2016; Sivagangabalan et al. 2011). As summarized above, a 3-hour exposure to ozone with moderate exercise did not lead to cardiovascular effects at 70 or 120 ppb in healthy older volunteers in the current study.

A recent report by the National Academies of Sciences (2017) reviewed the evidence provided by human controlled-exposure studies conducted by the U.S. EPA. The report concluded that those studies involving short-term exposures to ozone (O_3) "have contributed to clarification of exposure–response relationships and have been of critical importance for the NAAQS. … Those studies have provided … a basis for U.S. EPA's decision to move from a 1-hour to an 8-hour averaging time for the O_3 NAAQS level (concentration), and demonstrations of the importance of considering susceptibility factors and variability among individuals in human physiologic responses (such as changes in lung function) and biologic responses (such as increases in biomarkers of pulmonary inflammation) to exposure to ozone and other oxidant pollutants."

In its recent draft assessment of ozone and other oxidants, the U.S. EPA wrote that evidence "is sufficient to conclude that there is a causal relationship between short-term ozone exposure and respiratory health effects" (U.S. EPA 2019), consistent with its earlier conclusion (U.S. EPA 2013). EPA's determination of a causal association is based on new epidemiologic evidence, respiratory effects observed in human controlled-exposure studies at concentrations as low as 60 ppb ozone, and supportive evidence regarding biological plausibility from animal toxicology studies. In the 2019 draft Integrated Science Assessment, which cited results from MOSES 1, U.S. EPA also concluded that "When considered as a whole the evidence is suggestive of, but not sufficient to infer, a causal relationship between short-term exposure to ozone and cardiovascular effects," a different determination than that arrived at in 2013; this change was based on more limited evidence of effects, remaining uncertainty, and paucity of biological plausibility (U.S. EPA 2019). The MOSES study has provided additional important information about respiratory and cardiovascular effects at near-ambient concentrations that contributes to the scientific knowledge base. MOSES 2 has added confidence that the MOSES 1 results were not confounded by prior exposure to ambient air pollutants, including ambient ozone.

CONCLUSIONS

The Multicenter Ozone Study in oldEr Subjects was a large, well-conducted study in 87 healthy adults (55–70 years old). MOSES 1 provided the following important results: (1) there was no convincing evidence that a 3-hour exposure to near-ambient concentrations of 70 or 120 ppb ozone with moderate exercise resulted in statistically significant changes in cardiovascular endpoints in these healthy older adults; (2) short-term exposures at these relatively low ozone concentrations did lead to pulmonary effects; and (3) no susceptible subgroups could be identified in which ozone elicited cardiovascular effects that were not evident in the group as a whole. MOSES 2 showed that these results were not affected by the participants' immediate prior exposures to ambient air pollutants, providing additional confidence in the results. The MOSES Review Panel agreed with the main findings of the study and that the results support the conclusion that laboratory exposure to near-ambient levels of ozone can affect pulmonary function.

It remains possible that ozone may lead to cardiovascular effects in more susceptible individuals, following longer exposures, with greater levels of exertion, or in the presence of common ambient air pollutants. MOSES 2 presented some suggestive evidence that ambient air pollution exposure may be associated with changes in baseline levels of some cardiovascular and pulmonary biomarkers measured before the clinical visits. Ambient ozone concentrations were possibly associated with baseline measures of heart rate variability. Ambient fine particles, NO_2, and CO concentrations were associated with baseline measures of lung function. These results add to the body of evidence of changes in health outcomes associated with air pollutant exposures at the current — relatively low — ambient concentrations in the United States (see also Brauer et al. 2019; Dominici et al. 2019).

ACKNOWLEDGMENTS

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ABBREVIATIONS AND OTHER TERMS

AIC	Akaike Information Criteria
BAD	brachial artery diameter
BMI	body mass index
BP	blood pressure
BSA	body surface area
BTPS	body temperature and pressure, water saturated
CC16	club cell protein 16
CI	confidence interval
CO	carbon monoxide
CRP	C-reactive protein
CVD	cardiovascular disease
DBP	diastolic blood pressure
ECG	electrocardiogram
ED	emergency department
ET-1	endothelin-1
$FEF_{25–75}$	forced expiratory flow between 25% and 75% of FVC
FEV_1	forced expiratory volume in 1 second
FMD	flow-mediated dilatation
FVC	forced vital capacity
GSTM1	glutathione S-transferase mu 1
HF	high frequency power (0.15–0.40 Hz)
HR	heart rate
HRV	heart rate variability
iid	independent and identically distributed
IQR	interquartile range
IL-6	interleukin-6
LF	low frequency power (0.04–0.15 Hz)
ln	natural logarithm
MI	myocardial infarction
MOSES	Multicenter Ozone Study of oldEr Subjects
MOSES 1	Multicenter Ozone Study of oldEr Subjects Part 1
MOSES 2	Multicenter Ozone Study of oldEr Subjects Part 2
MP	microparticles
ms	millisecond
NAAQS	National Ambient Air Quality Standards
NN	normal-to-normal sinus beat (interval)
NNMAPS	National Morbidity, Mortality, and Air Pollution Study
NO_2	nitrogen dioxide
O_3	ozone
OR	odds ratio
PES	personal exposure sampler
PM	particulate matter
$PM_{2.5}$	particulate matter ≤ 2.5 μm in aerodynamic diameter
PMN	polymorphonuclear leukocytes (also referred to as “neutrophils”)
PMN%	percentage of polymorphonuclear leukocytes
ppb	parts per billion
ppm	parts per million
RH	relative humidity
RMSSD	root mean square of successive differences in normal-to-normal sinus beat intervals
RR	relative risk
SBP	systolic blood pressure
SD	standard deviation
SDNN	standard deviation of normal-to-normal sinus beat intervals
SO_2	sulfur dioxide
TNF-α	tumor necrosis factor-alpha
UCSF	University of California at San Francisco
UNC	University of North Carolina
URMC	University of Rochester Medical Center
U.S. EPA	U.S. Environmental Protection Agency
V_E	minute ventilation
VTI	velocity–time integral
vWF	von Willebrand factor

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