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REVIEW ARTICLE

Ozone exposure and systemic biomarkers: Evaluation of evidence for adverse cardiovascular health impacts

Julie E. Goodman[1], Robyn L. Prueitt[1], Sonja N. Sax[1], Daniella M. Pizzurro[1], Heather N. Lynch[1], Ke Zu[1], and Ferdinand J. Venditti[2]

[1]*Gradient, Cambridge, MA, USA and* [2]*Albany Medical College, Albany, NY, USA*

Abstract

The US Environmental Protection Agency (EPA) recently concluded that there is likely to be a causal relationship between short-term (< 30 days) ozone exposure and cardiovascular (CV) effects; however, biological mechanisms to link transient effects with chronic cardiovascular disease (CVD) have not been established. Some studies assessed changes in circulating levels of biomarkers associated with inflammation, oxidative stress, coagulation, vasoreactivity, lipidology, and glucose metabolism after ozone exposure to elucidate a biological mechanism. We conducted a weight-of-evidence (WoE) analysis to determine if there is evidence supporting an association between changes in these biomarkers and short-term ozone exposure that would indicate a biological mechanism for CVD below the ozone National Ambient Air Quality Standard (NAAQS) of 75 parts per billion (ppb). Epidemiology findings were mixed for all biomarker categories, with only a few studies reporting statistically significant changes and with no consistency in the direction of the reported effects. Controlled human exposure studies of 2 to 5 hours conducted at ozone concentrations above 75 ppb reported small elevations in biomarkers for inflammation and oxidative stress that were of uncertain clinical relevance. Experimental animal studies reported more consistent results among certain biomarkers, although these were also conducted at ozone exposures well above 75 ppb and provided limited information on ozone exposure-response relationships. Overall, the current WoE does not provide a convincing case for a causal relationship between short-term ozone exposure below the NAAQS and adverse changes in levels of biomarkers within and across categories, but, because of study limitations, they cannot not provide definitive evidence of a lack of causation.

Keywords

air pollution, biomarkers, causal framework, epidemiology, inflammation, mode of action, ozone, risk assessment, weight of evidence

History

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Address for correspondence: Julie E. Goodman, Gradient, 20 University Rd, Cambridge, MA 02138, USA. Tel: + (617) 395-5525. E-mail: jgoodman@gradientcorp.com

Introduction

The Clean Air Act mandates that the United States Environmental Protection Agency (EPA) sets health-based National Ambient Air Quality Standards (NAAQS) for six "criteria" air pollutants, including ozone. Ozone is a secondary pollutant, meaning that it is not directly emitted into the air from specific sources but is formed as a result of photochemical reactions between precursor gases, primarily nitrogen oxides (NO_x) and volatile organic compounds (VOCs), in the presence of ultraviolet (UV) rays. The formation and degradation of ozone are complex and depend on many factors, including the relative concentrations of precursor gases and meteorological factors (e.g., sunlight intensity and atmospheric mixing). The relative concentration of specific VOCs and NO_x is important for ozone formation, because under some conditions, formation of ozone is VOC-limited, whereas under other conditions, it is NO_x-limited (NRC 1991). Because NO_x is involved in both the formation and degradation of ozone, reducing NO_x increases ozone concentrations under some conditions. Also, ambient ozone concentrations have a distinct diurnal pattern because of their dependence on UV radiation. Typically, ozone concentrations begin increasing in the early morning hours, peak near mid-day, and decrease markedly at nighttime (Figure 1). Ambient ozone concentrations vary widely both spatially and temporally, and individual exposures to ozone vary as well (US EPA 2013). Mean background ozone concentrations range from 27 to 40 ppb across the US during the spring and summer, and can be higher than 60 ppb in the intermountain West (Zhang et al. 2011, Vingarzan 2004, US EPA 2013).

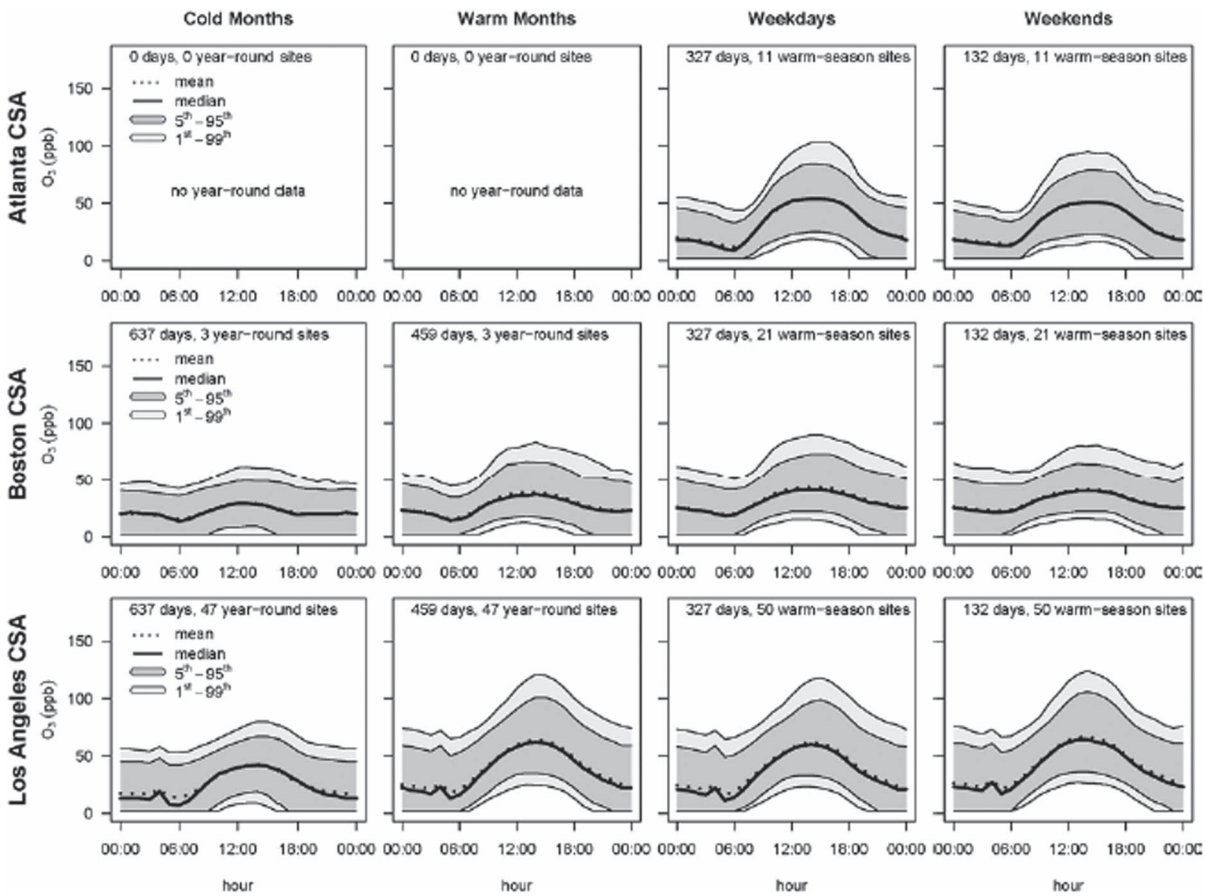

Figure 1. Diurnal patterns in 1-h average ozone concentrations. Data for Atlanta, Boston, and Los Angeles between 2007 and 2009. *Source:* US EPA, 2013.

Compliance with the NAAQS is determined by four elements: (1) the indicator (for photochemical oxidants, it is ozone); (2) the averaging time; (3) the numerical level or concentration; and (4) the statistical form (US EPA 2013). The numerical level and statistical form of the NAAQS determine its stringency. Any discussion of appropriate health-based NAAQS for ozone is incomplete without considering all four elements (Goodman et al. 2015). The ozone NAAQS established in 1971 used "photochemical oxidants" as an indicator and had an averaging time of 1 hour. It was set at 0.08 parts per million (ppm), equivalent to 80 ppb, and the form specified that the level was not to be exceeded more than 1 hour per year. In 1979, the indicator and the associated measurement methodology were changed from "photochemical oxidants" to ozone. The 1-hour averaging time was retained and the numerical level changed to 0.12 ppm, with attainment defined when the expected number of days per calendar year with maximum hourly average concentrations greater than 0.12 ppm is equal to or less than one. In 1997, the ozone NAAQS was again changed, with the ozone indicator retained, the averaging time changed from 1 hour to 8 hours, the level reduced from 0.12 ppm to 0.08 ppm (corresponding to 0.084 ppm, by rounding convention), and attainment defined as "the 3-year average of the annual fourth-highest daily maximum 8-hour average" (US EPA, 1997). The shift in the averaging time reflected a growing body of evidence for health effects associated with 6- to 8-hour exposures below the level of the 1-hour NAAQS at 0.12 ppm. The shift in the form to a concentration-based ozone standard that allowed multiple exceedances was viewed as being protective of public health and providing increased stability of the NAAQS for achieving compliance. In 2008, the ozone NAAQS was revised so that the annual fourth-highest daily maximum 8-hour concentration of ozone, averaged over three years, should not exceed 0.075 ppm (75 ppb).

Several health-effect endpoints have been associated with ozone exposure in the epidemiology literature. In recent analyses, ozone has been suggested as a potential causal factor for cardiovascular disease (CVD). For example, in its most recent review of the ozone health-effects literature for the re-evaluation of the ozone NAAQS, the EPA concluded that there was "likely to be a causal relationship" between short-term ozone exposure and cardiovascular (CV) "effects" (i.e., morbidity and mortality), including CVD (US EPA 2013). CVD is the leading cause of morbidity and mortality in the US (Lloyd-Jones et al. 2010). Nearly 1 in 3 US adults has some form of CVD (Montgomery and Brown 2013). There are several major risk factors for CVD, including age, male gender, hypertension, smoking, sedentary behavior, elevated low-density lipoprotein (LDL) cholesterol, family history of CVD, obesity, and diabetes. Age is the most important risk factor for developing CVD, with an approximate tripling of risk with each increasing decade of life (Finegold et al. 2013).

To provide perspective for evaluating the CV mortality risks of ozone, Petito Boyce et al. (2015) reviewed multiple risk factors that have been evaluated for their association with CVD mortality. For long-term risk factors, the highest relative risks were for diabetes, smoking, sedentary behavior, family history of CVD, exercise, and socioeconomic status, which were in the range of 1.47–2.86. In contrast, Lipsett et al. (2011) and

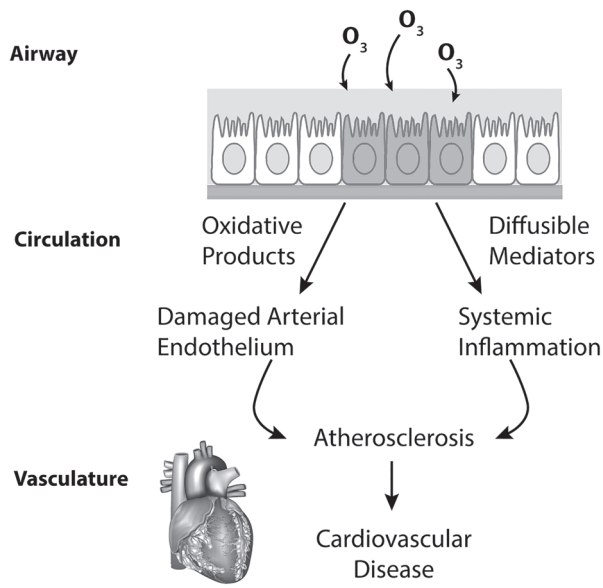

Figure 2. Proposed MoA for ozone-induced cardiovascular disease. Inhaled ozone (O_3) reacts with biomolecules in the fluid lining the lungs, damaging airway epithelial cells and generating oxidative products that enter the circulation and directly damage the arterial endothelium. The damaged epithelial cells release a number of diffusible mediators into the circulation that can activate a systemic inflammatory response. The combination of damaged arterial endothelium and systemic inflammation promotes atherosclerosis, the underlying cause of cardiovascular disease.

Jerrett et al. (2009) found no statistically significant effect of chronic ozone exposure on CVD mortality when results were adjusted for particulate matter ($PM_{2.5}$) exposure and when other risk factors such as demographic characteristics, smoking history, alcohol use, and diet were considered. Short-term risk factors such as stress from high-pressure work deadlines, episodes of physical or sexual activity, and acute anger had relative risks well over 2.0. In contrast, a meta-analysis of 39 studies evaluating the association between short-term exposure to ozone and CVD mortality found a central tendency relative risk ratio of 1.0111, which was not statistically significant (Bell et al. 2005). Overall, Petito Boyce et al. (2015) did not find evidence for a causal role of ozone in CVD mortality, in stark contrast to validation of the causal role of well-known risk factors.

Despite the lack of an established causal role of ozone in CVD, several modes of action (MoAs) have been proposed as explanations for the associations observed between ozone exposure and CV morbidity and mortality in some epidemiology studies. Ozone reacts directly with respiratory tract lining fluids and is not transported to extrapulmonary sites (Hatch et al. 1994, Medinsky 1996), but it is possible that ozone reaction products from the respiratory tract enter the circulation (Figure 2). One proposed MoA is the generation of oxidative products from the reaction of ozone with lipids or cellular membranes in the lung, which are released into the circulation and contribute to systemic effects (Chuang et al. 2009, US EPA 2013). A similar pathway that is often cited involves the release of diffusible mediators from ozone-induced lung injury (such as cytokines and growth factors) that then enter the circulatory system and initiate or propagate a systemic inflammatory response, contributing to atherosclerosis (Cole and Freeman 2009, US EPA 2013).

Atherosclerosis, the underlying cause of CVD, is a progressive disease of the arterial wall characterized by formation of

plaque. Coronary artery disease (CAD), the most common type of CVD diagnosed in the US, results from atherosclerosis in the coronary arteries that supply oxygen-rich blood to the heart muscle. There is increasing evidence that atherosclerosis begins early in life, and its occurrence and rate of development are influenced by multiple risk factors over the course of decades (Lloyd-Jones et al. 2010).

The molecular pathway to atherosclerosis is complex and involves factors related to inflammation, oxidative stress, coagulation, vasoreactivity, and lipid/glucose metabolism (Figure 3). Specifically, the endothelial cells that line the inner surface of arteries are subject to injury from many insults, including oxidative stress (Zakynthinos and Pappa 2009). Injured endothelial cells express adhesion molecules that facilitate their attachment to inflammatory cells (i.e., white blood cells such as monocytes) (Libby et al. 2011). The injured cells also secrete chemoattractant cytokines that mediate the migration of the monocytes into the subendothelial space (i.e., the intima) of the artery (Libby et al. 2011, Moore et al. 2013). Once in the artery wall, the monocytes differentiate into macrophages that engulf LDL particles and transform into lipid-laden foam cells (Libby et al. 2011, Moore et al. 2013). Foam cells constitute the fatty streak, which is the first recognizable progenitor of an advanced atherosclerotic plaque (Zakynthinos and Pappa 2009). The foam cells produce reactive oxygen species, tissue factor procoagulants, and cytokines that recruit inflammatory cells, resulting in further uptake of LDL, as well as the stimulation of smooth muscle cell proliferation and the development of a collagenous fibrous cap over the core of the plaque (Libby et al. 2011).

Atherosclerotic plaques protrude into the vessel lumen and cause stenosis, or narrowing of the arteries, resulting in reduced blood flow to tissues (i.e., ischemia). Plaques can also be physically disrupted, and the presence of inflammatory cells can hasten this process (Zakynthinos and Pappa 2009). Once disrupted, the procoagulant material in the core of the plaque is exposed to coagulation proteins in the circulating blood, which triggers thrombosis or blood clot formation that can block the artery, leading to major adverse clinical events (Insull 2009, Libby et al. 2011).

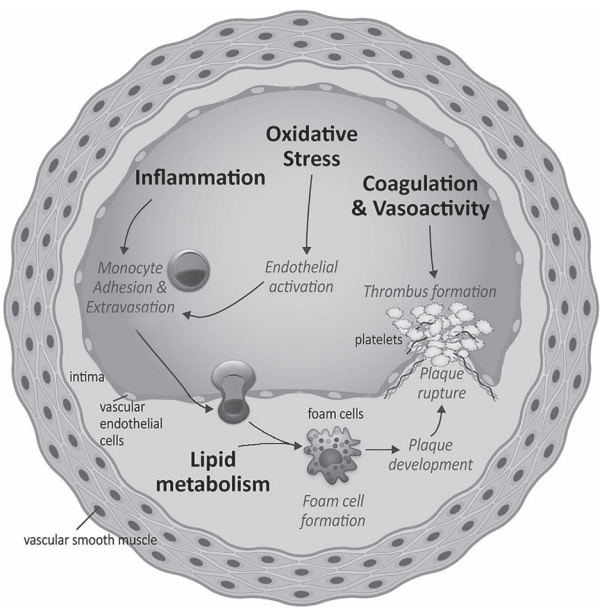

Figure 3. Molecular pathway to atherosclerosis. Atherosclerosis develops over decades and may be acutely exacerbated by factors associated with many biochemical pathways, including inflammation, oxidative stress, coagulation, vasoreactivity, and lipid/glucose metabolism, as described in the text.

The clinical expression of atherosclerosis is highly variable. After a prolonged "silent" period, atherosclerosis most often becomes clinically manifest in mid- to late life. The clinical expression of atherosclerosis can be subacute, such as the development of stable angina (i.e., chest pain); a dramatic acute event, such as myocardial infarction (MI; i.e., a heart attack) or an acute stroke; or the most devastating manifestation, sudden cardiac death (Libby 2001). By contrast, some individuals may never experience clinical manifestations of their disease.

As noted above, the EPA concluded that there was "likely to be a causal relationship" between short-term ozone exposure and CV effects, despite noting "inconsistent" evidence for many of the CV morbidity endpoints examined (US EPA 2013). We conducted an independent, systematic evaluation of the same evidence and concluded that there is no convincing case for a causal relationship in humans, but limitations of the available studies preclude definitive conclusions regarding a lack of causation (Goodman et al. 2014). Because our conclusions differed from those of the EPA, it was of interest to conduct a detailed evaluation of ozone exposure effects on levels of circulating biological markers, or biomarkers, which are associated with the molecular pathway to atherosclerosis described above, to further examine the plausibility of the proposed MoAs. If ozone is a causal factor in CVD, one would expect to see changes in biomarker levels induced by ozone exposure that are consistent with atherosclerosis development and increased risk of CVD.

In the present analysis, we assess whether it is plausible that ambient levels of ozone could contribute to CVD by impacting biomarkers related to the acceleration or exacerbation of atherosclerosis. We apply the principles of a weight-of-evidence (WoE) framework described by Goodman et al. (2013), referred to herein as the "Goodman WoE framework," in a systematic review of the available studies that assessed changes in levels of biomarkers with short-term (< 30 day) exposure to ozone. The Goodman WoE framework incorporates the critical steps for a scientifically sound systematic review and is based on best practices from a survey of more than 50 WoE frameworks, including the EPA's NAAQS causal framework (Rhomberg et al. 2013).

Methods

To evaluate the effects of ozone on biomarker levels, we applied the general principles of the Goodman WoE framework (Goodman et al. 2013), which consists of four phases. In Phase 1, we defined the causal question, study quality criteria, and inclusion/exclusion criteria for selecting the biomarkers and studies to evaluate. In Phase 2, we extracted study characteristics into tables, then categorized studies based on the study quality criteria established in Phase 1, using a crude quantitative scoring method. In Phase 3, we integrated the evidence for each biomarker category within and across realms of evidence (i.e., epidemiology, controlled human exposure, and experimental animal). Within each realm, we evaluated the individual study results and consistency of results across studies for each biomarker. In Phase 4, we categorized the causal relationship between short-term ozone exposure and adverse changes in biomarker levels based on the WoE conclusions from Phase 3.

Causal question and study selection

In Phase 1, we defined the principal question for our evaluation: Does short-term ozone exposure below the current NAAQS cause CVD *via* the release of biomarkers into the bloodstream that then accelerate or exacerbate atherosclerosis? To be consistent with EPA's definition, we defined short-term exposure as <30 days in duration (US EPA 2013).

We selected the biomarkers to include in our evaluation based on clinical experience and a review of several comprehensive assessments of ozone and adverse CV outcomes (for example, US EPA 2013, Goodman et al. 2014, Prueitt et al. 2014). In the final analysis, we included 41 biomarkers in the categories of inflammation, oxidative stress, coagulation, vasoreactivity, and lipid/glucose metabolism (Table 1). As these categories are associated with the molecular pathway to atherosclerosis, for simplicity we refer to the total group of selected biomarkers herein as "atherosclerosis-related" biomarkers, even though alterations in the levels of each of these biomarkers may not be risk factors or prognostic indicators of atherosclerosis or CVD.

We conducted two PubMed searches to identify studies published through January 8, 2014, one with and one without "ozone" as a MeSH term. The first search included the following search terms: [Specific biomarker][1] + [Ozone (MeSH)] + [NOT pulmonary[ti] OR respiratory[ti] OR lung OR lungs OR bronchial[ti] OR fev[ti] OR bal[ti]].[2] The second search included the following search terms: [Specific biomarker] + [Ozone (text)] + [NOT ozone (MeSH)].

We included epidemiology studies, controlled human exposure studies, and experimental animal studies that evaluated the effects of ozone on measured biomarkers in plasma, serum, blood, urine, or heart tissue. We included only English-language studies that evaluated ozone exposure for durations of <30 days.

We excluded studies that focused on pulmonary endpoints; studies measuring biomarkers in tissues other than the blood or heart (e.g., brain, skin, kidney); studies that evaluated ozonated blood, ozone oxidative preconditioning, or the use of ozone for a therapeutic purpose; studies that were not published in English; *in vitro* studies; studies in non-mammalian species (e.g., plants); observational studies evaluating indoor ozone exposure; studies evaluating ozone exposure for a duration ≥ 30 days; and studies using a non-inhalation route of exposure.

Development and evaluation of study quality criteria

In Phase 1, we developed separate sets of criteria to evaluate study quality consistently across each realm of evidence based on those used in previous study quality evaluations (e.g., Goodman et al. 2014, Prueitt et al. 2014), as well as the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (Kilkenny et al. 2010) and other international research guidelines, such as those of the Organisation for Economic Co-operation and Development (OECD) and the World Health Organization (WHO) (OECD 1998, WHO 2009). In Phase 2, we assessed the strengths and limitations of each study based on these study quality criteria, and also used these criteria to roughly categorize studies as high- or low-quality.

Specifically, we assigned each study a score of -1 or 1 for each criterion. To do this, we made decisions on cutoffs for a positive versus negative score that were subjective in some cases, but we explain these decisions below, and we applied each criterion consistently across all studies. We then calculated an overall score by summing the scores for all criteria. The overall scores are only a crude measure of quality; a study may be high-quality based on one criterion but low-quality based on another. Although some of the criteria may be of greater importance than others, we did not assign weights to the categories because it is impossible to know how much each quality criterion may impact study results. Rather, we used our crude scoring system to group the studies into two tiers, to indicate whether a study has more strengths (positive qualities) or more limitations (negative qualities). Studies in Tier I have an overall score >0 and studies in Tier II have an overall score ≤ 0. We considered Tier I studies to be of generally higher quality relative to Tier II studies, but our assessment of the evidence in Phase 3 considers the strengths and limitations of each individual study and how they may impact the interpretation of results. In our discussion of the studies, we also address additional factors not included in our scoring system that may impact the interpretation or relevance of individual study results.

Epidemiology studies

We evaluated the epidemiology studies and assigned each a score (1 or -1) in the following study quality categories:

Study design. We considered longitudinal analyses that took into account both between- and within-subject variation by measuring biomarkers repeatedly in the same subjects to be the most robust for making causal inferences and assigned a study design score of 1. In cross-sectional studies, within-subject variation is not accounted for and can undermine the validity of the results. Thus, we assigned a study design score of -1 to all cross-sectional studies.

Study size. The majority of the epidemiology studies did not perform any study power calculation to assess whether the number of participants was sufficient to observe effects. Therefore, we used two cutoffs for study size: ≥ 100 participants for cross-sectional studies; and ≥ 50 participants and ≥ 100 measurements among the participants for longitudinal analyses (Goodman et al. 2014, Prueitt et al. 2014). We assigned a score of 1 to studies that met these criteria and –1 to those that did not.

Selection bias. We considered the risk of selection bias likely to be low in studies that clearly indicated that the selection of participants was unrelated to ozone exposure (e.g., geographically well-defined populations or a random sample of geographically well-defined populations), and we assigned a selection bias score of 1 to these studies. We assigned a selection bias score of -1 to all studies for which we judged the risk of selection bias likely to be high. The risk of selection bias was likely to be high in studies with inclusion criteria based on availability of air monitoring data or distance to an air monitoring station because inclusion in the study was directly linked to data availability, which could also be associated with outcomes (e.g., if monitors are placed in certain areas based on expected maximal concentrations, such as near important sources of pollution). The risk of selection bias was

[1]See Table 1 for search terms used for each biomarker.
[2][ti] = title; [MeSH] = medical subject headings (PubMed search term).

Table 1. Biomarkers identified for evaluation.

Inflammation	Oxidative stress	Coagulation/Vasoreactivity	Lipid/Glucose metabolism
C-Reactive Protein (CRP)	2,3-dehydroxybenzoic acid (DHBA)	Atrial Natriuretic Factor (ANF)	Apolipoprotein A1 (ApoA1)
Interleukin (IL)-6	8-hydroxy-2′-deoxyguanosine (8-OHdG)	D-dimer	Apolipoprotein B (ApoB)
IL-10	8-isoprostaglandins-F2α (8-iso-PGF)	Endogenous Thrombin Potential (ETP)	Hemoglobin A1c (HbA1c)
IL-2	Ferric Reducing Ability of Plasma (FRAP)	Endothelin-1 (ET-1)	High-density Lipoprotein (HDL) Cholesterol
IL-8	Malondialdehyde (MDA)	Fibrinogen	Lipoprotein-associated Phospholipase A2 (Lp-PLA2)
IL-1β	Superoxide dismutase (SOD)	Plasminogen	Low-density Lipoprotein (LDL) Cholesterol
Intracellular Adhesion Molecule-1 (ICAM-1)		Plasminogen Activator Inhibitor-1 (PAI-1)	Total Cholesterol
Lymphocytes		Platelet Aggregation	Triglycerides
Neutrophils		Thrombin Peak Height (TPH)	
Tumor Necrosis Factor-α (TNF-α)		Thrombomodulin	
Total Homocysteine (tHcy)		Tissue Factor (TF)	
Vascular Cell Adhesion Molecule-1 (VCAM-1)		Tissue-type Plasminogen Activator (tPA)	
White Blood Cells		Vascular Endothelial Growth Factor (VEGF)	
		von Willebrand Factor (vWF)	

also likely to be high if participants were recruited from a single or from a few clinics, hospitals, or other institutions, because the inclusion of these participants may have been related to socioeconomic status factors that correlate with ozone exposure. Several studies relied on volunteers; self-selection can increase the risk of selection bias because an individual's decision to participate may be related to exposure, outcome, or both. For studies with a low response rate (which may increase the likelihood of a differential response between cases/controls or exposed/non-exposed) or high loss to follow-up (>20%), the risk of attrition bias may be high if the non-response rate and/or loss to follow-up are related to either the exposure or the outcome under study.

Exposure assessment. Exposure measurement error is common in epidemiology studies of ozone because most rely on centrally located air monitors and use measurements of ambient concentrations as a proxy for individual exposure. We considered studies that restricted the study population to participants residing within 10 kilometers (km) of air monitoring stations or relied on mathematical models such as inverse distance weighted models to estimate average ozone concentrations of a smaller area unlikely to have considerable exposure measurement error; we assigned an exposure assessment score of 1 to these studies. For studies that used area-level (such as city- or county-wide) ozone concentrations, we judged that the extent of exposure measurement error was likely to be larger and assigned a score of − 1 to these studies.

Quality assurance/Quality control (QA/QC) protocols. Some biomarkers are only stable when frozen, so sample handling, processing, and storage methods can affect their measured levels (Pearson et al. 2003, Zhou et al. 2010). Because samples from epidemiology studies are collected from study subjects and processed at various times and often analyzed in different laboratories, we considered whether the studies reported and implemented appropriate QA/QC protocols for sample collection and storage. We assigned a QA/QC score of 1 to studies that did and − 1 to those that did not.

Assay reproducibility. The accuracy and precision of biomarker assays can impact the interpretation of results. Given the observational nature of epidemiology studies and the potential for considerable inter-laboratory and inter-assay variation, we assessed whether authors provided quantitative measures of reproducibility for the bioassay measurements. For this category, we assigned a score of 1 to studies that reported good reproducibility of the bioassays (e.g., coefficient of variation ≤10%, or intraclass correlation coefficient >75%), and a score of –1 to studies that did not.

Statistical analyses. We evaluated whether studies conducted appropriate statistical analyses to evaluate the effects of ozone exposure on CV-related biomarkers. We considered linear regression (for continuous outcome) or logistic regression (for binary outcome) to be appropriate for cross-sectional studies, and linear mixed-effects models to be appropriate for longitudinal studies; thus, we assigned a statistical analysis score of 1 to studies that used such models. For studies that did not use these models, we assigned a score of − 1.

Co-pollutants. Confounding by co-pollutants is likely to occur in epidemiology studies of ozone because concentrations of air pollutants tend to be highly correlated with one another and the outcome of interest. This may be particularly true for ozone and particulate matter (PM), especially for particles < 2.5 μm ($PM_{2.5}$) (Barath et al. 2013, US EPA 2009). We assigned a score of 1 to studies that included bi- or multi-pollutant models and a score of − 1 to those that only considered single-pollutant models.

Confounding. We considered five categories of potential confounders: demographic, lifestyle, temporal, meteorological, and other. Demographic confounders included age, sex, race/ethnicity, community/area, education, income, marital status, employment, and public assistance. Lifestyle confounders included body mass index (BMI), smoking, waist circumference, physical activity, alcohol consumption, healthy eating index, and multivitamin and aspirin use. Temporal confounders

included time of day, date, day of the week, day of the year, weekday, month, season, year, and long-term time trend. Meteorological confounders included temperature, humidity, apparent temperature, pressure, cloud cover, and presence of precipitation. Other confounders included medical history of CVD, diabetes, chronic respiratory disease, and other chronic diseases or health conditions; family history of premature coronary events; lipidology, glucose, and vitamins; blood pressure; contraceptive or hormone use; medication use; gestational week; and parity. We judged studies that considered at least one factor from each of the demographic, lifestyle, temporal, and meteorological categories to have adequately adjusted for confounders and assigned a control for confounding score of 1. Otherwise, we assigned a score of − 1.

Lag time. Because the timing of exposure to ozone that could result in an adverse outcome is unknown, we considered whether studies investigated different lag times for ozone exposure. We assigned a lag time score of 1 to studies that evaluated multiple lag times and − 1 to studies that only considered a single lag.

Sensitivity analyses. We considered whether analyses were carried out to assess the sensitivity of study findings to various assumptions. We assigned a sensitivity analysis score of 1 to studies that evaluated ozone and CV outcomes using alternative statistical model assumptions or alternative statistical models altogether, and a score of − 1 to studies that did not conduct any sensitivity analyses.

Controlled human exposure studies

We evaluated the controlled human exposure studies and assigned each a score (1 or − 1) in the following study quality categories:

Study design. We considered crossover studies to be the most appropriate study design because each participant serves as his or her own control, thus eliminating bias from differences between individuals (e.g., weight, height, age, health status). In addition, studies that were randomized (i.e., the sequence of air versus ozone exposure was assigned randomly) minimized the impact of factors that may be associated with the timing of exposure (e.g., if the time of month impacts biomarker levels, independent of exposure). We assigned studies that met these criteria a study design score of 1; if not, we assigned a study design score of − 1.

Study size. Clinical differences in response to exposure may be small, so we assigned a study size score of 1 to studies that conducted power calculations and reported sufficient power to detect small effects of ozone. We assigned a study size score of − 1 to studies that did not report study power.

Participant selection. If participants were recruited randomly from a diverse area through methods such as a website, newspaper, or random calling, we considered selection of participants likely unrelated to ozone exposure or biomarker levels and assigned a participant selection score of 1. If participants were volunteers or from a single institution, we considered the risk of selection bias as likely to be higher and assigned a participant selection score of − 1.

Blinding. Although several studies reported double blinding (i.e., both participants and investigators were blinded to exposure status), ozone has an odor threshold of 30 ppb or less (NLM 2014), and all studies used exposures of at least 100 ppb. Still, blinding of investigators to exposure status can reduce the potential for bias; therefore, we assigned studies a score of 1 in this category if authors reported blinding of data entry and/or data analysis personnel. We assigned a score of − 1 to studies with no blinding.

Exposure assessment. We assigned an exposure assessment score of 1 to studies that reported a clear description of maintenance of ozone exposure (i.e., type of ozone generation equipment), environmental conditions (e.g., temperature and humidity), and methods of ozone generation, delivery, and monitoring (i.e., equipment used and whether monitoring of ozone concentration was continuous). We assigned an exposure assessment score of − 1 to studies that lacked detailed descriptions of any of these parameters.

QA/QC protocols. We assigned a QA/QC score of 1 to studies that implemented and reported detailed protocols for sample collection and storage, and − 1 to those that did not.

Assay reproducibility. We assigned a score of 1 to studies that specified the assays or kits (and their origins) used for the measurement of biomarkers with enough detail so that the reader could reproduce the analysis. We assigned a score of − 1 when these details were absent, or in cases where a non-standardized or commercially available method was referenced but not described.

Statistical analysis. We assigned a statistical modeling score of 1 to studies that used appropriate statistical methods to account for the correlation between outcome measurements, as measurements within individuals are correlated and should not be treated as independent. We considered appropriate methods to be mixed-effects models, analysis of variance with repeated measures, and paired t-tests for comparison of pre- and post-exposure values. We assigned a score of − 1 to studies that used tests we considered to be inadequate (e.g., t-tests without consideration of covariance) or had missing data or unclear statistical methods.

Experimental animal studies

We evaluated the experimental animal studies and assigned each a score (1 or − 1) in the following study quality categories:

Randomization. We assigned a score of 1 to studies that explicitly stated whether animals were randomized into treatment or control groups and − 1 to studies that did not.

Study size. We assigned a study size score of 1 to studies that provided a clear description of the different treatment groups and included at least five animals of one sex per group, as per US EPA (1998) guidelines for acute inhalation studies, unless otherwise justified (e.g., with a power calculation). We assigned score of − 1 if these conditions were not met.

Exposure assessment. We assigned an exposure assessment score of 1 to studies that explicitly described the measures taken to ensure accuracy and consistency of the ozone exposure throughout the exposure period (e.g., continuous

monitoring of ozone concentration), type of exposure method used (e.g., chamber or nose-only), maintenance of adequate environmental conditions, and density of animals in each chamber (to minimize effects of animal surface area or volume on exposure concentration). We assigned a score of − 1 if these parameters were not described clearly in the study.

Animal housing and husbandry. Information on animal housing and husbandry is integral to a reliable animal study. We assigned a score of 1 to studies that included a description of the animals used (e.g., where purchased or bred), methods for feeding and housing of animals, treatment conditions (including ethical guidelines), acclimation period, age of animals, and sacrifice methods. We assigned an animal housing and husbandry score of − 1 if more than one of these details was missing or if no information was provided.

Controls. To evaluate the use of controls, we assigned a score of 1 to studies that compared groups of animals exposed to ozone to a control group exposed to filtered air (FA). We assigned a score of − 1 to studies that did not use an FA control group or did not compare the ozone-exposed group to the FA control group. For example, this included studies that exposed control animals to "room" or "ordinary air" (which may have contained contaminants that could confound results) or compared biomarker levels in ozone-exposed animals to baseline biomarker levels.

QA/QC protocols. We assigned a QA/QC score of 1 to studies that provided details on sample collection, handling, and storage methods, and − 1 if these details were not reported.

Assay reproducibility. We assigned a score of 1 to studies that specified the assays or kits (and their origins) used for the measurement of biomarkers with enough detail so that the reader could reproduce the analysis. We assigned a score of − 1 when these details were absent, or in cases in which a non-standardized or commercially available method was referenced but not described.

Attrition bias. We assigned an attrition bias score of 1 to studies that provided details of study-related deaths. We assigned a score of − 1 in this category if no information on study-related deaths was provided or if it could not be derived from other study information.

Statistical analysis. We assigned a statistical analysis score of 1 to studies that used appropriate statistical methods, including methods to account for possible correlations between repeated measures, and included information regarding standard errors or standard deviations, baseline/control results, and data for all relevant time points in the presentation of results. We assigned a score of − 1 to studies that used inappropriate statistical tests or had missing data.

Evaluation and integration of evidence

In Phase 3, we evaluated and integrated the evidence within and across realms. First, we assessed individual study results within each realm, as well as the consistency of results across studies for each biomarker category. For this assessment, we considered strength of association, temporality, internal consistency, biological plausibility, random error (i.e., chance), and exposure–response relationships, when feasible. Second, we integrated the data across realms of evidence, considering strength of association, consistency of associations, coherence, biological gradient, biological plausibility, temporality, specificity, confounding, bias, and clinical relevance of effects. We compared alternative accounts of the evidence and formulated WoE conclusions, considering all studies but assigning more weight to the studies we judged to be of higher quality in Phase 2 (i.e., Tier I studies).

Assessment of the causal relationship

In Phase 4, we applied the WoE conclusions from Phase 3 to categorize the potential causal relationship between short-term ozone exposure and adverse changes in levels of atherosclerosis-related biomarkers. We relied on the categories of causal determination proposed in the Institute of Medicine (IOM) report *Improving the Presumptive Disability Decision-making Process for Veterans* (IOM 2008). The IOM framework has four categories of causal determination: "Sufficient," "Equipoise and above," "Below equipoise," and "Against"; use of the IOM framework's four-level categorization scheme is consistent with WoE best practices (Goodman et al. 2013).

Literature search results

We initially identified 1,247 articles in PubMed; 1,128 of these did not meet the inclusion criteria described in the section "Causal question and study selection." The final list of studies included 19 epidemiology, 10 controlled human exposure, and 23 experimental animal studies (Figure 4).

Evaluation of study quality

We evaluated each study based on specific study quality criteria (Section "Study quality criteria development and evaluation"); below, we provide a brief summary of study characteristics and quality evaluation for each of the studies considered.

Epidemiology studies

We identified 19 epidemiology studies for inclusion in our evaluation (Table 2). The populations evaluated in these studies included mostly healthy adults, although some studies evaluated elderly populations (Delfino et al. 2010, Bind et al. 2012), populations with a history of CVD (Delfino et al. 2010, Bruske et al. 2011), pregnant women (Lee et al. 2011), or children (Poursafa et al. 2011). Studies were conducted worldwide, with locations in the US, Canada, China, Taiwan, Italy, the United Kingdom, Germany, the Netherlands, Israel, and Iran. Ozone exposure metrics varied across studies and included hourly, 8-h, 24-h, multi-day, and monthly averages.

Table 2 presents the study quality characteristics and quality scores for the epidemiology studies. Of the 19 studies we identified, 11 were cross-sectional and eight were longitudinal. The majority of studies had adequate study size and QA/QC protocols, employed appropriate statistical models, and evaluated multiple lags; therefore, we assigned them scores of 1 in each of these categories. However, selection bias was a

potential issue in most studies, as most investigators recruited participants from a single institution or excluded participants based on the availability of air monitoring data. In addition, the majority of epidemiology studies did not report reproducibility measures for bioassays, adjust for co-pollutants, or perform sensitivity analyses; thus, we assigned most studies a score of −1 for these categories. Overall, based on our study quality evaluation, we classified 12 epidemiology studies as Tier I and seven as Tier II (Table 2).

It should be noted that although we considered the Tier I epidemiology studies to be of higher quality, there were still methodological limitations in these studies that could affect the interpretation of their results. For example, the vast majority of the epidemiology studies evaluated a number of statistical associations between multiple air pollutants, lag times, and biomarkers, but none of the studies adjusted for multiple comparisons in their analyses. Because of this lack of adjustment, it is possible that some of the observed associations are attributable to chance (dos Santos Silva 1999).

Controlled human exposure studies

We identified 10 relevant controlled human exposure studies for inclusion in our evaluation (Table 3). Eight of the studies were crossover studies conducted in chambers; the other two studies (Bergamaschi et al. 2001, Strak et al. 2013) were semi-experimental studies with ambient ozone exposure. All 10 studies had similar sample sizes (16–31 participants), with the exception of the study by Buckley et al. (1975), which had only six participants, and Chen et al. (2007), which had 120 participants. The participants were generally young adults aged 18–28 years, on average; however, Buckley et al. (1975) did not report the ages of participants in their study. Ozone concentrations in the studies ranged from 120 to 500 ppb. Exposure duration ranged from 2 to 5 h, and all but two studies (Brook et al. 2009, Urch et al. 2010) required participants to engage in intermittent exercise while exposed. Exercise increases ventilation as well as oral breathing (versus nasal breathing), resulting in the delivery of a larger dose of ozone to the airways (Hatch et al. 2013). Several studies measured biomarkers at more than one time point (e.g., immediately after exposure cessation and 24 h post-exposure).

Table 3 presents the study quality characteristics and quality scores for the controlled human exposure studies. Of the 10 studies we identified, five were randomized crossover designs and five were either semi-experimental or crossover designs without randomization. The majority of studies adequately generated and monitored ozone exposure and adequately described QA/QC methods (i.e., for blood collection and storage conditions) and assay details (i.e., type of assay, kit, source); therefore, we assigned the majority of studies a score of 1 in these categories. Half of the studies blinded laboratory technicians to the exposure status of participants, so we assigned a blinding score of 1 to these studies. Half of the studies used adequate statistical methods that adjusted for multiple comparisons, and we assigned these studies a score of 1 for this category. For the majority of studies, we judged selection bias to be possible because the investigators recruited participants from a single institution (e.g., a university) or did not report how subjects were recruited; thus, we assigned most studies a score of −1 for this category. The exceptions were the studies by Brook et al. (2009) and Urch et al. (2010), which used recruitment methods that included a diverse set of potential participants. Most of the studies also had small study sizes and no power calculations to ensure sufficient power to detect any effects, so we assigned a score of −1 for study size to the

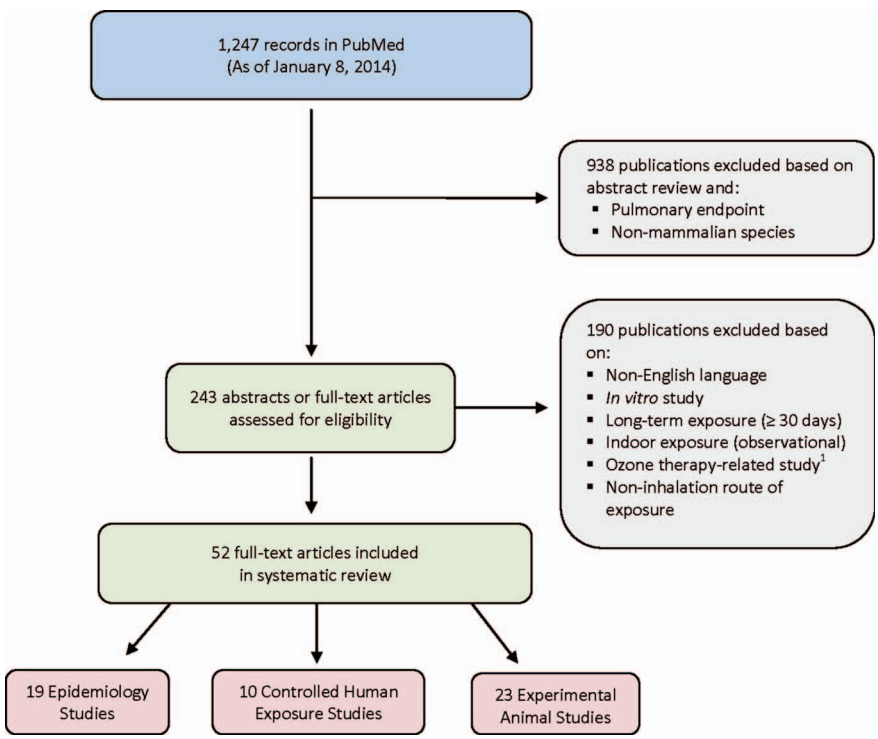

Figure 4. Literature search strategy. [1]Ozone therapy-related studies include those pertaining to ozonated blood, ozone oxidative preconditioning, or the use of ozone for therapeutic purposes.

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Table 2. Study quality ratings for epidemiology studies.

					Outcome assessment				Statistical analysis							
									Confounders adjusted for							
Reference	Study design	Selection bias	Study size	Exposure Measurement	QA/QC protocol	Reproducibility of measurements	Model type	Multi-pollutant	Demographic	Lifestyle	Temporal	Meteorological	Other	Multiple lags	Sensitivity analysis	Total score
Bruske et al. (2011)	Longitudinal	Possible (only picked survivors with less severe MI)	200	Local-level (≤10 km)	Protocol reported for sample collection and storage	CV% = 2.07%	Linear mixed effects models	None	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
Quality score	1	−1	1	1	1	1	1	−1	1					1	1	7
Chuang et al. (2007)	Longitudinal	Possible (recruitment from a single university)	76	Local-level (monitors within 1 km)	Protocol reported for sample collection and storage	NR	Linear mixed effects models	Sulfate	Yes	Yes	Yes	Yes	None	Yes	No	
Quality score	1	−1	1	1	1	−1	1	1	1					1	−1	5
Lee et al. (2011)	Cross-sectional	Unlikely	1696	Zip-code level	Protocol reported for sample collection and storage	CV% = 7%	Logistic regression	None	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
Quality score	−1	−1	1	−1	1	1	1	−1	1					1	1	5
Zhang et al. (2013)	Longitudinal	Possible (subjects selected from a single hospital)	125	Local-level (<10 km)	NR	NR	Linear mixed effects models	PM, CO, NO_2, SO_2, EC, OC, SO_4^{2-}	Yes	Yes	Yes	Yes	None	Yes	Yes	
Quality score	1	−1	1	1	−1	−1	1	1	1					1	1	5
Bind et al. (2012)	Longitudinal	Unlikely	704	City-level	NR	NR	Linear mixed effects models	None	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
Quality score	1	1	1	−1	−1	−1	1	−1	1					1	1	3
Chen et al. (2007)	Cross-sectional	Possible (recruitment from a single university)	120	Local-level (IDW modeling)	Protocol reported for sample collection and storage	CV% <10%	Linear regression	None	Yes	Yes	None	None	Yes	Yes	No	
Quality score	−1	−1	1	1	1	1	1	−1	−1					1	−1	1
Chuang et al. (2010)	Cross-sectional	Unlikely (national survey samples of Taiwanese population)	7,578	Local level (≤10 km)	NR	NR	Generalized additive models	None	Yes	Yes	Yes	Yes	None	Yes	No	
Quality score	−1	1	1	1	−1	−1	1	−1	1					1	−1	1
Delfino et al. (2010)	Longitudinal	Possible (volunteers)	60	Local-level (at each retirement community)	Protocol reported for sample collection and storage	NR	Linear mixed effects models	None	Yes	Yes	Yes	Yes	None	No	No	
Quality score	1	−1	1	1	1	−1	1	−1	1					−1	−1	1
Pekkanen et al. (2000)	Cross-sectional	Possible (loss to follow-up in the original cohort)	7,205	City-level	Reported for sample collection and storage	CV% = 7.7%	Linear regression/ Logistic regression	None	Yes	Yes	Yes	Yes	None	Yes	No	
Quality score	−1	−1	1	−1	1	1	1	−1	1					1	−1	1
Ren et al. (2011)	Cross-sectional	Unlikely	320	City-level	Protocol reported for sample collection and storage	CV% <20%	Linear regression	None	Yes	Yes	Yes	Yes	Yes	Yes	No	
Quality score	−1	1	1	−1	1	−1	1	−1	1					1	−1	1

(*Continued*)

Table 2. (Continued)

					Outcome assessment				Statistical analysis							
									Confounders adjusted for							
Reference	Study design	Selection bias	Study size	Exposure Measurement	QA/QC protocol	Reproducibility of measurements	Model type	Multi-pollutant	Demographic	Lifestyle	Temporal	Meteorological	Other	Multiple lags	Sensitivity analysis	Total score
Rich et al. (2012)	Longitudinal	Possible (subjects selected from a single hospital)	125	Local-level (<10 km)	NR	NR	Linear mixed effects models	None	Yes	Yes	Yes	Yes	None	Yes	No	
Quality score	1	−1	1	1	−1	−1	1	−1	1					1	−1	1
Rudez et al. (2009)	Longitudinal	Possible (healthy volunteers)	40	Local-level (distance unspecified)	Protocol reported for sample collection and storage	NR	Generalized additive models	None	Yes	Yes	Yes	Yes	None	Yes	Yes	
Quality score	1	−1	−1	−1	1	−1	1	−1	1					1	1	1
Baccarelli et al. (2007a)	Cross-sectional	Possible (exclusion of area without air monitoring data)	1,213	Region-level	Protocol reported for sample collection and storage	NR	Linear regression	None	Yes	Yes	Yes	Yes	Yes	Yes	No	
Quality score	−1	−1	1	−1	1	−1	1	−1	1					1	−1	−1
Baccarelli et al. (2007b)	Cross-sectional	Possible (exclusion of area without air monitoring data)	1,214	Region-level	Protocol reported for sample collection and storage	NR	Linear regression	None	Yes	Yes	Yes	Yes	Yes	Yes	No	
Quality score	−1	−1	1	−1	1	−1	1	−1	1					1	−1	−1
Liao et al. (2005)	Cross-sectional	Possible (exclusion of subjects without air monitoring data)	8,639	County-level	Protocol reported for sample collection and storage	NR	Linear regression	None	Yes	Yes	Yes	Yes	Yes	Yes	No	
Quality score	−1	−1	1	−1	1	−1	1	−1	1					1	−1	−1
Poursafa et al. (2011)	Cross-sectional	Unlikely (random samples of children and adolescents)	118	Local-level (<1 km)	Protocol reported for sample collection and storage	NR	Linear regression/ Logistic regression	None	Yes	Yes	None	None	None	No	No	
Quality score	−1	1	1	1	1	−1	1	−1	−1					−1	−1	−1
Steinvil et al. (2008)	Cross-sectional	Possible (exclusion of subjects living > 11 km from air monitoring stations, low retention rate)	3,659	City-level	NR	NR	Linear regression	PM_{10}, SO_2, NO_2	Yes	Yes	Yes	Yes	Yes	Yes	No	
Quality score	−1	−1	1	−1	−1	−1	1	1	1					1	−1	−1
Thompson et al. (2010)	Longitudinal	Possible (participants from controlled exposure studies)	45	City-level	Protocol reported for sample collection and storage	NR	Linear mixed effects models	None	Yes	Yes	Yes	Yes	Yes	Yes	No	
Quality score	1	−1	−1	−1	1	−1	1	−1	1					1	−1	−1
Schwartz (2001)	Cross-sectional	Possible (only subjects in urban area with air monitoring data)	~20,000	Local-level	Reported for sample collection and storage	NR	Linear mixed effects models	None	Yes	Yes	None	None	None	No	No	
Quality score	−1	−1	1	1	1	−1	1	−1	−1					−1	−1	−1

CO carbon monoxide, CO_2 carbon dioxide, *CV%* coefficient of variation, *EC* elemental carbon, *IDW* inverse distance weighting, *MI* myocardial infarction, NO_2 nitrogen dioxide, *NR* not reported *OC* organic carbon, *PM* particulate matter, *QA* quality assurance, *QC* quality control, SO_2 sulfur dioxide, SO_4^{2} sulfate.

majority of the studies. Overall, based on our evaluation of study quality, we classified four controlled human exposure studies as Tier I and six as Tier II (Table 3).

Experimental animal studies

We identified 23 experimental animal studies for inclusion in our evaluation (Table 4). The majority of experimental animal studies were conducted using healthy, post-weaning-aged rats or rats near the end of their lifespan. Other studies were conducted in mice (Fujimaki et al. 1987, Feng et al. 2001, Chuang et al. 2009) or guinea pigs (Vaughan et al. 1984, Chhabra et al. 2010). All studies were conducted *in vivo*, with the exception of the study by Perepu et al. (2010), which was an *ex vivo* study on isolated rat hearts from ozone-exposed or unexposed rats subjected to ischemia and reperfusion before the biomarker levels were measured. We considered the *in vivo* studies to be more relevant than the *ex vivo* study. The exposure durations were either acute (2, 4, or 8 h) or subacute (2–4 weeks, or $<$ 30 days) (OECD 2009, US EPA 2013). The majority of studies evaluated only one exposure concentration, although some evaluated two or more. Ozone exposure concentrations ranged from 120 to 12,500 ppb across studies.

Table 4 presents the study quality characteristics and quality scores for the experimental animal studies. The majority of studies provided adequate descriptions of the exposure environment and used an appropriate number of animals per group; therefore, we assigned a score of 1 for exposure assessment and study size to the majority of studies. Most studies did not explicitly state whether exposure assignment was randomized and did not provide information regarding attrition bias, so we assigned most of the studies a score of -1 for these categories. Several studies used an inappropriate control group (i.e., ozone-exposed animals were not compared to FA-exposed controls) and many of these same studies used inappropriate statistical methods; thus, we assigned these studies a score of -1 for these categories. Overall, based on our study quality evaluation, we classified 17 experimental animal studies as Tier I and six as Tier II (Table 4).

Evaluation of study results

In the following sections, we summarize and evaluate the results of the epidemiology, controlled human exposure, and experimental animal studies of short-term ozone exposure and atherosclerosis-related biomarkers. For each study type, we considered strength of association, temporality, internal consistency, biological plausibility, random error, and exposure–response relationships within each category of biomarkers. In addition, we considered the clinical relevance of statistically significant changes in biomarker levels (i.e., whether the changes are associated with disease), as well as the consistency of results across studies for each biomarker category. Because few biomarkers were examined in more than one study in each realm of investigation, comparisons of the effects of ozone on specific biomarkers across studies were not feasible in some cases. Prior to the evaluation of study results, a brief overview of the various biomarkers that we included for evaluation is provided in each section below.

Biomarkers of inflammation

Of the many biomarkers relevant to atherosclerosis that are associated with inflammation, the majority are proinflammatory and should increase in concentration if systemic inflammation is induced. The most well-studied inflammatory biomarker for CVD risk is C-reactive protein (CRP), an acute-phase–response protein that is a general marker of inflammation. Circulating concentrations of CRP can increase up to 50,000-fold within 6 h during acute inflammatory conditions, such as infection (Gilstrap and Wang 2012).

Several prospective studies in healthy participants have reported that elevated CRP levels correlate with higher risk for future CV morbidity and mortality (as reviewed by Zakynthinos and Pappa 2009). Unlike other acute-phase–response proteins, levels of CRP remain stable over long periods of time in the absence of new inflammatory stimuli, with no diurnal variation (Pearson et al. 2003, Ridker et al. 2002, Zakynthinos and Pappa 2009). In addition, assay techniques for measurement of circulating CRP levels are reliable and sensitive (Zakynthinos and Pappa 2009). The Centers for Disease Control and Prevention (CDC) and the American Heart Association (AHA) have stated that patients without known CVD but who are at intermediate risk (10–20% risk of CAD over 10 years) may benefit from CRP measurement as a way of assessing their current CVD status.

Production of CRP occurs in the liver in response to proinflammatory cytokines. Cytokines are secreted factors involved in mediating inflammatory and immune responses (Zhou et al. 2010, Stoner et al. 2013). Proinflammatory cytokines include interleukins (ILs) such as IL-1, IL-2, IL-6, and IL-8, as well as tumor necrosis factor-α (TNF-α). IL-6 can also have anti-inflammatory activity at low physiological levels and in response to muscle contraction (Sarwar et al. 2009, Stoner et al. 2013). Elevated IL-6 levels may be predictive of mortality from CVD and are associated with adverse outcomes in patients with acute coronary syndromes (Stoner et al. 2013). IL-10 is an anti-inflammatory cytokine that inhibits the synthesis of some proinflammatory cytokines (Zhou et al. 2010), and has been associated with a lower risk for MI and death in acute coronary syndrome patients (Stoner et al. 2013). Once produced, cytokines are rapidly immobilized by high affinity receptors on neighboring cells, which may limit their usefulness as a surrogate endpoint when measured in the circulation (Stoner et al. 2013). Certain cytokines, such as IL-1 and TNF-α exhibit distinct diurnal variations, and levels of many cytokines are affected by dietary intake, exercise, stress, and trauma (Zhou et al. 2010). In addition, many cytokines have a short half-life and begin to degrade once blood is drawn, so samples should be processed quickly and kept frozen, to decrease the likelihood of artifacts in measurements (Zhou et al. 2010).

The intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are additional biomarkers of interest because they are expressed by injured endothelial cells in the early stages of atherosclerosis. They mediate the attachment of circulating white blood cells to the endothelium and their migration across the endothelial barrier. In a prospective study of patients with CAD, ICAM-1 and VCAM-1 levels were associated with future mortality, but in a model that controlled for all soluble adhesion and inflam-

Table 3. Study quality ratings for controlled human exposure studies.

			Study size		Outcome assessment					
Reference	Study design	Selection of participants	*N*	Sample size calculation	QA/QC protocol	Measures of reproducibility	Exposure type & maintenance	Statistical analysis	Blinding	Total score
Brook et al. (2009)	Cross-over, randomized	Advertisements (newspapers, fliers, websites) used locally and selection followed blinded randomization protocol	31	Power calculations (80% power)	Blood sample collection and storage reported	Assay kits and source provided	Chamber, well maintained	Paired t-test,mixed effects model; no adjustment for multiple comparisons	Personnel blinded	
Quality score	1	1	1		1	1	1	−1	1	6
Urch et al. (2010)	Cross-over, randomized	Recruited from University of Toronto and Toronto area	23	None	Blood sample collection and storage conditions reported	ELISA kits and source provided	Chamber, well maintained 1	ANOVA and multiple regression	Technician blinded	
Quality score	1	1	−1		1	1	1	1	1	6
Liu et al. (1997)	Cross-over, randomized	Recruitment method not stated, but exclusion/inclusion criteria specified	16	None	Storage of plasma samples reported	Measurement methods well described	Chamber, well maintained	One-way ANOVA followed byStudent-Newman-Keuls test	Technician blinded	
Quality score	1	−1	−1		1	1	1	1	1	4
Liu et al. (1999)	Cross-over, randomized	Recruitment method not stated, but exclusion/inclusion criteria specified	20	None	Blood sample collection and storage conditions reported	Measurement methods well described	Chamber, well maintained	Two-way ANOVA or two-way repeated measures ANOVA followed by Newman-Keuls test	Technician blinded	
Quality score	1	−1	−1		1	1	1	1	1	4
Gong et al. (1998)	Cross-over, not randomized	NR	6–10	None	Blood sample collection and storage conditions reported	Measurement methods and kits well described	Chamber; well maintained	ANOVA, as a within-subjects factor	Technician blinded	
Quality score	−1	−1	−1		1	1	1	−1	1	0
Buckley et al. (1975)	Cross-over, not randomized	NR	6	None	Blood sample collection and storage conditions reported	Specific methods not well described	Generation and chamber environmental conditions well described; unclear what tech. used for monitoring but chamber was monitored	Paired t-tests	Not blinded	
Quality score	−1	−1	−1		1	−1	1	1	−1	−2

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Devlin et al. (2012)	Cross-over, randomized	Identified potential subjects through website, newspaper advertising, and targeted calls to qualified subjects who had completed other US EPA studies	23	None	NR	ELISA kits and source provided	Chamber, no mention of maintenance	Paired t-test, and mixed-effects models; not appropriate to log-transform normally distributed data; no adjustment for multiple comparisons	Outcome assessors not said to be blinded	
Quality score	1	1	−1		−1	1	−1	−1	−1	−2
Strak et al. (2013)	Cross-over, semi-experimental	All recruited from a university and all lived on campus	31	None	Blood sample collection and storage conditions reported	ELISA kits and source provided	Outdoors, in five diverse locations	Mixed linear regression, comprehensive	No blinding of technicians/ researchers reported	
Quality score	−1	−1	−1		1	1	−1	1	−1	−2
Chen et al. (2007)	Chamber (Served as own controls but no air exposure.)	Students recruited from 1 university	120	None	Blood collection and storage conditions reported, and QA/QC experiments conducted to determine if artifactual formation of 8-iso-PGF levels in plasma occurred	Coefficients of variations calculated for measurements	Chamber, no mention of maintenance	Student t-tests followed by multivariable analyses	NR	
Quality score	−1	−1	−1		1	1	−1	−1	−1	−4
Bergamaschi et al. (2001)	Semi-experimental	NR	24	None	NR	Method detailed briefly, but not in depth; references other paper	Ambient ozone continuously measured via UV photometric analyzer; cones, varied	Paired t-test, Pearson's correlation analysis mentioned for other endpoints, but unclear what was used for relevant CV biomarker	Not blinded	
Quality score	−1	−1		−1	−1	−1	−1	−1	−1	−8

8-iso-PGF 8-isoprostaglandins-$F_{2\alpha}$*ANOVA*, analysis of variance, *ELISA* enzyme-linked immunosorbent assay, *N* number, *NR* not reported, *QA* quality assurance, *QC* quality control, *US EPA* Environmental Protection Agency, *UV* ultraviolet.

Table 4. Study quality ratings for experimental animal studies.

	Study design					Outcome assessment				
Reference	Exposure assignment (Randomization)	Exposure environment	Experimental groups	Experimental animals, housing, and husbandry	Compared to appropriate control	Sample storage and handling	Assay specifics	Presence of attrition bias	Statistical analysis	Total score
Mole et al. (1985)	Randomized	O_3 monitored and calibrated; Sufficient exposure details given	Satisfactory	Sufficient detail provided	Yes	Sufficient detail provided. (blood sampling times, fasting periods, sample handling, storage)	Standards used well-referenced methods for analyses; Sufficient detail	Reported. No deaths/signs of toxicity	ANOVA used to appropriately account for multiple comparisons	
Quality score	1	1	1	1	1	1	1	1	1	9
Kodavanti et al. (2011)	NR	O_3 generated with gas-phase titration diluter; O_3 concentration monitored continuously	Satisfactory	Sufficient detail provided	Yes	Sufficient detail provided. (blood collection methods, tissue processing well detailed)	Detail on specific kits and antibodies used; Well-referenced methods; Methods for data analysis well described	Based on number of animals in beginning and end of analyses, no deaths occurred	One-way ANOVA; Some used Kruskal-Wallis ANOVA; Appropriate *post hoc* tests used; Clearly defined where appropriate	
Quality score	−1	1	1	1	1	1	1	1	1	7
Liu et al. (1996)	Randomized	O_3 concentration continuously monitored by sampling at the manifold; Many details on exposure scenario	Only 4 animals in exposed group	Sufficient detail provided	Yes	Sufficient detail provided. (anesthesia method described, heparinized tubes, storage details)	Well-referenced methods; A lot of detail on quantification methods and assay parameters	Reported. Only deaths in senescent animals; Not considered treatment-related	Two-way ANOVA followed by Newman-Keuls.	
Quality score	1	1	−1	1	1	1	1	1	1	7
Martinez-Campos et al. (2012)	NR	O_3 measured in control (<0.05 ppm); O_3 maintained with servomechanism in chamber	Satisfactory	Sufficient detail provided.	Yes	Sufficient detail provided. (anesthesia method described, heparinized arterial blood samples, sample processing described)	Specific kits and their origin provided (although no other detail).	Based on number of animals in beginning and end of analyses, no deaths occurred	One-way ANOVA and multiple comparison *post hoc* tests (Tukey's) or two-way ANOVA with Bonferroni	
Quality score	−1	1	1	1	1	1	1	1	1	7
Wang et al. (2013)	Randomized	O_3 produced by O_3-generator using UV light combined with air; O_3 concentration measured every 2 min.; Probes at height of breathing zone; O_3 in control <0.04 ppm	Satisfactory	Sufficient detail provided	"Ordinary Air"-exposed controls; Unclear if filtered to remove contaminants	Sufficient detail provided. (sacrifice methods, sample collection and storage well detailed)	Specific kits and their origin provided	Reported. No deaths.	2-way ANOVA, followed by Tukey's multiple comparisons	
Quality score	1	1	1	1	−1	1	1	1	1	7

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Bouthillier et al. (1998)	Randomized	O_3 was produced from pure oxygen in a silent arc generator, continuously monitored by UV spectrometry; Varied ±5% from target concentration	Satisfactory	No age or acclimation period provided	Yes	Sufficient detail provided. (blood/ plasma collection and storage methods)	Specific kits and their origin provided; A lot of detail on biochemical and cytological analyses provided	NR	Multiway ANOVA, followed by Tukey's test	
Quality score	1	1	1	−1	1	1	1	−1	1	5
Chuang et al. (2009)	NR	O_3 continuously monitored; Details provided on turnover rates, O_3 production, and filtered air conditions.	Satisfactory	Sufficient detail provided	Yes	Sufficient detail provided (Sacrifice and tissue collection methods, storage details)	Specific kits/ antibodies and their origin provided; Well-referenced methods	NR	ANOVA to test null hypothesis, followed by Student-Newman-Keuls for group comparisons	
Quality score	−1	1	1	1	1	1	1	−1	1	5
Jakubowski et al. (2004)	Randomized	O_3 measured by iodometric method; Chamber air exchanged half-way through exposure duration	Satisfactory	Sufficient detail provided.	Unknown if exposed to filtered air or just untreated	Sufficient detail provided. (blood collection methods, storage details)	Specific kits and their origin provided; Well-referenced methods	Based on number of animals in beginning and end of analyses, no deaths occurred	Student's t-test used; multiple comparisons not accounted for	
Quality score	1	1	1	1	−1	1	1	1	−1	5
Perepu et al. (2010)	NR	O_3 monitored and regulated; Methods O_3 in control air 0.0 ppm reported	Satisfactory	Sufficient detail provided	Yes	Sufficient detail provided. (tissue collection and storage details)	Detail/description of methods; Referenced methods; Kit details provided	NR	One-way ANOVA followed by Newman-Keuls test	
Quality score	−1	1	1	1	1	1	1	−1	1	5
Perepu et al. (2012)	NR	O_3 monitored and regulated; Methods reference Perepu et al. (2010) (O_3 in control air 0.0 ppm reported)	Satisfactory	Sufficient detail provided	Yes	Sufficient detail provided. (tissue collection and storage details)	Detail/description of methods; Referenced methods; Kit details provided	NR	One-way ANOVA, followed by Newman-Keuls test	
Quality score	−1	1	1	1	1	1	1	−1	1	5
Sanchez-Gonzalez et al. (2004)	NR	Animals kept under filtered air at all times when not in exposure chambers to ensure $O_3 < 0.05$ ppm; O_3 monitored/ maintained by servomechanisms	Only 4 animals/txt group	Sufficient detail provided	Yes	Sufficient detail provided. (Blood collection methods, storage details)	Assay details (duplicate measurements) and specific kits/their origin provided	Based on number of animals in beginning and end of analyses, no deaths occurred	Data between groups tested with Student-Newman-Keuls test; Blood markers tested by two-way ANOVA	
Quality score	−1	1	−1	1	1	1	1	1	1	5

(*Continued*)

Table 4. (Continued)

	Study design					Outcome assessment				
Reference	Exposure assignment (Randomization)	Exposure environment	Experimental groups	Experimental animals, housing, and husbandry	Compared to appropriate control	Sample storage and handling	Assay specifics	Presence of attrition bias	Statistical analysis	Total score
Kadiiska et al. (2013)	NR	Methods from Kadiiska et al. (2011) state that O_3 concentration was monitored and gave sufficient detail of chamber conditions	Satisfactory	No age or acclimation period reported	Methods from Kadiiska et al. (2011) state filtered room air	Sufficient detail provided. (blood and urine sample collection and storage details)	Detail/description of methods; Referenced methods; Kit details provided; Detection limits and interassay CVs given where appropriate	NR	ANOVA used appropriately; Pooled estimates of error; Stabilized variances by logarithmic transformation	
Quality score	−1	1	1	−1	1	1	1	−1	1	3
Sethi et al. (2012)	NR	O_3 monitored and regulated; O_3 measured in control air (0.0 ppm reported)	Satisfactory	No acclimation period; Age of rats given as "adult," but actual age not given; No information on where animals were purchased or bred	Yes	Sufficient detail provided. (tissue collection and storage details)	Detail/description of methods; Referenced methods; Kit details provided	NR	One-way ANOVA, followed by Newman-Keuls test	
Quality score	−1	1	1	−1	1	1	1	−1	1	3
Chhabra et al. (2010)	NR	O_3 generator; Concentration continuously monitored; Measures to avoid CO_2 accumulation	Satisfactory	No details on acclimation; No details of age of animals; Minimal detail on housing conditions; No guidelines/ ethics	Yes	Insufficient detail on sample storage. (no details of storage temperature provided for plasma samples, no way to determine if samples were processed immediately)	Well-referenced methods, but no detail in paper; Not known if they are commercialized kits or what their origins are otherwise	Based on number of animals in beginning and end of analyses, no deaths occurred	ANOVA and Bonferroni used; Also used Kruskal-Wallis and Mann Whitney U non-parametric tests; Paired t-test/ Wilcoxon signed rank to compare baseline and sensitization; Clearly defined where appropriate	
Quality score	−1	1	1	−1	1	−1	−1	1	1	1

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Cretu et al. (2010)	NR	O_3 concentration regulated; Sufficient detail on exposure methods	Satisfactory	Sufficient detail provided	No filtered air-exposed control; "Maintained in a normal atmosphere"	Sufficient detail provided. (blood collection, processing, and storage conditions)	Sufficient detail on assay protocol and reagents; references for methods used	NR	Fisher test followed by t-test used; Poorly described statistical analysis; Inappropriate statistical methods used; Poorly presented statistical result; Inconsistent figure and text	
Quality score	−1	1	1	1	−1	1	1	−1	−1	1
Thomson et al. (2006)	NR	According to a previous publication, O_3 generated through silent arc generator and monitored by ultraviolet spectrophotometry at the nose-only inhalation ports after sampling through a Teflon filter	Satisfactory	No acclimation period or age	Animals were compared to their own pre-exposure values when exposed to "clean air."	Sufficient detail provided. (animal sacrifice, plasma collection and storage conditions)	Referenced HPLC methods; Specific kits and origin provided where appropriate	NR	Two-way ANOVA and Tukey's test for multiple comparisons	
Quality score	−1	1	1	−1	−1	1	1	−1	1	1
Vesely et al. (1994a)	NR	Sufficient detail; O_3 concentration monitored	Satisfactory	Sufficient detail provided	Controls only exposed to "room air"; Unclear if filtered to remove contaminants	Sufficient detail provided. (sacrifice and tissue collection methods)	Assay details provided (timing, temperature, all steps, triplicate measurements); Interassay coefficients of variation determined for the different measured markers	Not reported. (Though methods note that none of the animals became ill during the study)	ANOVA, but no *post hoc* tests mentioned	
Quality score	−1	1	1	1	−1	1	1	−1	−1	1

(*Continued*)

Table 4. (Continued)

	Study design					Outcome assessment				
Reference	Exposure assignment (Randomization)	Exposure environment	Experimental groups	Experimental animals, housing, and husbandry	Compared to appropriate control	Sample storage and handling	Assay specifics	Presence of attrition bias	Statistical analysis	Total score
Feng et al. (2001)	NR	Measures taken to maintain and measure constant O_3 concentration	Satisfactory	Only 2-day acclimation period and limited other housing and husbandry details	"Air"-exposed controls; Unclear if filtered to remove contaminants	Sufficient detail provided (animal sacrifice, sample collection and storage conditions)	Assay details and kit details/ origins provided; Well-referenced methods	NR	One-way ANOVA and Student's t-test used; No post-hoc test mentioned; Unclear when ANOVA used instead of t-test; For endpoint of interest, data is only presented for one time point with no explanation as to why others were excluded	
Quality score	−1	1	1	−1	−1	1	1	−1	−1	−1
Nachtman et al. (1988)	NR	O_3 monitored; "Chamber concentration within 10% target values"	Satisfactory	Age not given; Unknown "quarantine" time	"Air"-exposed controls; Unclear if filtered to remove contaminants	Insufficient detail on sample storage. (Not explicitly stated if samples analyzed immediately or stored)	Details on instrumentation and methods provided; Origin of reagents; Well-referenced methods	Reported. (Mortality included as primary endpoint)	Student's t-test and Duncan's Multiple Range test used; For relevant endpoints, data is poorly presented: only in text and very incomplete (*e.g.*, no quantitative data for one endpoint, no p-values); Statistical methods are also unclear	
Quality score	−1	1	1	−1	−1	−1	1	1	−1	−1
Vaughan et al. (1984)	NR	O_3 monitored and air replenished 13x/h; Chamber volume and air-flow parameters given	Satisfactory	Sufficient detail provided	No filtered air-exposed control	Insufficient detail on sample storage and collection. (No details on use of EDTA/ heparin tubes for stabilization, or no storage	Insufficient detail on sample assay methods and conditions	Reported. 2/19 males and 3/21 females died during 2-week exposure period	Student's t-test used; Multiple comparisons not accounted for; Groups compared for statistical analysis are unclear	
Quality score	−1	1	1	1	−1	−1	−1	1	−1	−1

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Takatori (1975)	NR	O_3 generated by "high voltage, electric spark Ozone generator"; Concentration "determined several times a day"	Satisfactory	Missing age, acclimation period, guidelines, and sufficient detail on housing conditons.	No filtered air control; Used pre-O_3 exposure as control.	Insufficient detail on sample storage	Insufficient detail on assay methods and conditions	NR	Student's t-test used; Multiple comparisons not accounted for; No SD given for higher dose results	
Quality score	−1	1	1	−1	−1	−1	−1	−1	−1	−5
Bobb and Fairchild (1967)	NR	No exposure details given (*e.g.*, method used or if O_3 concentration was monitored)	No report of number of animals/ group	NR	No	Insufficient information on sample collection and storage	Referenced method used for white blood cell count and justified method choice	NR	None given; No SD/ SE given with results	
Quality score	−1	−1	−1	−1	−1	−1	1	−1	−1	−7
Fujimaki et al. (1987)	NR	O_3 monitored but few details given; No O_3 concentration in control; Exposure duration is unclear	Satisfactory	No acclimation period; Insufficient detail on animal conditions; No guidelines/ ethical standards	Unknown treatment of control.	Insufficient detail on sample collection methods. (no information on animal sacrifice, limited information on tissue processing/ cell collection	Limited detail on assay conditions and methods (filter size and other conditions not provided)	NR	Student's t-test used; Multiple time points analyzed but multiple comparisons not accounted for	
Quality score	−1	−1	1	−1	−1	−1	−1	−1	−1	−7

ANOVA analysis of variance, *CO_2* carbon dioxide, *CV* cardiovascular, *EDTA* ethylenediaminetetraacetic acid, *HPLC* high-performance liquid chromatography, *NR* not reported, *O_3* ozone, *ppm* parts per million, *SD* standard deviation, *SE* standard error, *UV* ultraviolet.

matory markers, only VCAM-1 was independently associated with the risk of future CV events (Stoner et al. 2013).

Increased circulating levels of total homocysteine (tHcy) are considered an independent risk factor for CVD (Baccarelli et al. 2007a, Zhang et al. 2014). tHcy is a non-protein amino acid synthesized from methionine, and it has been shown to have adverse effects on vascular endothelium and smooth muscle. Inflammation is a determinant of hyperhomocysteinemia, and plasma tHcy levels are associated with increased CRP and IL-6 (Baccarelli et al. 2007a). Circulating levels of tHcy can vary with diet; for example, supplementation with folic acid can lower tHcy levels (Libby 2001). Although elevated tHcy has been associated with an increased risk of CVD in several studies, meta-analyses indicate that the strength of association decreased with increasing study quality (Lewington et al. 2012).

In addition to circulating proteins, inflammatory cells such as lymphocytes, neutrophils, or total white blood cell counts have been measured as risk factors for CVD. Increases in circulating levels of such cells are general markers of inflammation that may be attributed to infection or other conditions unrelated to CVD.

The results of studies that evaluated the effects of short-term ozone exposure on biomarkers of inflammation are shown in Table 5.

Epidemiology studies

Eight circulating biomarkers were assessed in epidemiology studies that evaluated the association between short-term ozone exposure and inflammation (CRP, white blood cell count, IL-6, neutrophil count, lymphocyte count, ICAM-1, VCAM-1, and tHcy). All of these biomarkers are proinflammatory and should increase in response to ozone exposure, if ozone induces systemic inflammation. The results of these studies are described below and have been summarized in Table 5; detailed study results can be found in Supplementary Table 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371.

CRP. The effects of short-term ozone exposure on levels of CRP were investigated in four Tier I studies and one Tier II study. The Tier I studies reported increases in CRP associated with short-term ozone exposures in at least one statistical model, but there were some inconsistencies in the direction of change for similar lag times across studies. Three of these studies were longitudinal analyses (Chuang et al. 2007, Rudez et al. 2009, Bind et al. 2012) and one was a cross-sectional analysis (Lee et al. 2011). Chuang et al. (2007) reported that a 17.9-ppb increase in 24–h average ozone concentrations (a lag of 0 days) was associated with a non-statistically significant increase in CRP of 74.3%, and a 16-ppb increase in 2-day average ozone concentrations (a lag of 0–1 day) was associated with a statistically significant increase in CRP of 120% in healthy young adults in Taiwan. In contrast, Rudez et al. (2009) reported a non-significant decrease in CRP levels with a 21-ppb increase in 24-h average ozone concentrations at a lag of 1 day in healthy adults in the Netherlands. They also reported non-significant increases in CRP, of 3.7% and 5.9%, for a 21-ppb increase in ozone concentrations at lags of 2 and 3 days, respectively. Bind et al. (2012) conducted a longitudinal analysis in older men in the US and reported a statistically significant increase in CRP, of 10.7%, with increased ozone concentrations at a lag of 0 days. The authors reported smaller, non-significant CRP increases with various multiple-day lags (see Supplementary Table 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371). Lee et al. (2011) reported that interquartile range (IQR) increases in short-term ozone exposures (8.7, 8.2, and 7.7 ppb for 8-day, 22-day, and 29-day average concentrations, respectively) were associated with moderately increased odds (odds ratios [ORs] ranged from 1.05 to 1.49) of having a high CRP level (defined by the authors as ≥ 8 g/mL) in healthy pregnant women, but these risk estimates were not statistically significant despite the large number of participants in this study.

The Tier II study, a large-scale, cross-sectional analysis of healthy individuals in Israel, reported that an increase of 15 ppb ozone was associated with small increases in CRP levels at a lag of 0 or 1 day, and small decreases at later lags of up to 7 days (Steinvil et al. 2008). None of these changes were statistically significant.

Together, the epidemiology studies of CRP reported associations with ozone exposure that were inconsistent in direction across studies, even at similar lag times. None of these studies employed multi-pollutant models to evaluate whether the associations between ozone and CRP were confounded by co-pollutants. In addition, the clinical relevance of the reported changes in CRP levels is unclear. The magnitudes of the reported increases in CRP levels were relatively small across studies, although the significant increase reported by Chuang et al. (2007) was much larger (120%). The mean CRP level for participants in the study by Chuang et al. (2007) was reported to be 0.8 mg/L, with a 95% confidence interval (CI) of 0.73–0.87 mg/L; serum levels of CRP below 1 mg/L are considered low-risk for CVD (Montgomery and Brown 2013). Thus, these increased CRP levels may not be clinically relevant with respect to adverse CV outcomes.

White blood cell count. The effects of short-term ozone exposure on white blood cell count were investigated in two Tier I and three Tier II studies. Among the Tier I studies, a longitudinal analysis in healthy, non-smoking young adults in China reported that a 25.4-ppb increase in 24-h average ozone concentration was associated with a 1.4% decrease in white blood cell counts at a lag of 0 days and small increases at later lags of up to 6 days, but the changes were not statistically significant (Rich et al. 2012). In a re-analysis of the same data, Zhang et al. (2013) used bi-pollutant models and showed that ozone exposure at a lag of 5 days was associated with small increases (1.7–3.3%) in white blood cell counts. The increases were statistically significant when adjusted for nitrogen dioxide (NO_2) or sulfur dioxide (SO_2), but were reduced and not significant when adjusted for PM, carbon monoxide (CO), elemental carbon (EC), organic carbon (OC), or sulfate, indicating potential confounding by these co-pollutants. The clinical relevance of the increases are also unclear, as they are small changes, and the mean white blood cell count for the participants during the highest exposure period was 5.40 (0.15 standard error) $\times 10^9$/L, which is well within the normal reference range of 4.5×10^9/L to 11×10^9/L (Leikin and Paloucek 2008).

Table 5. Results for biomarkers of inflammation.

Studies	CRP	ICAM-1	IL-1	IL-10	IL-6	IL-8	Lymphocytes	Neutrophils	TNF-a	Total Homocysteine (tHcy)	VCAM-1	White Blood Cell Count
Direction of Adverse Change	↑	↑	↑	↓	↑	↑	↑	↑	↑	↑	↑	↑
Epidemiology												
Chuang et al. (2007)	**↑**											
Rudez et al. (2009)	↑↓											
Delfino et al. (2010)					↓							
Lee et al. (2011)	↑											
Bind et al. (2012)	**↑**	**↑↓**									**↑↓**	
Rich et al. (2012)												↑↓
Zhang et al. (2013)							**↑***	↑↓				**↑***↓
Liao et al. (2005)												↓
Baccarelli et al. (2007a)										**↑↓**		
Steinvil et al. (2008)	↑↓											↑↓
Thompson et al. (2010)					**↓↑**							
Schwartz (2001)												↓
Controlled human exposure												
Brook et al. (2009)	↑↑							↑↑	– –			↑↑
Urch et al. (2010)†					– –‡				– –‡			
Devlin et al. (2012)	**↑↑**		**↑↑**		↑↓	**↑↑**		↑↓	↓↑			
Experimental animal												
Jakubowski et al. (2004)	↑											
Perepu et al. (2010)				**↓**					**↑**			
Kodavanti et al. (2011)							↑					↑
Perepu et al. (2012)				**↓**					**↑**			
Sethi et al. (2012)									**↑**			
Wang et al. (2013)	↑				↓							
Bobb and Fairchild (1967)							↓	↑				
Fujimaki et al. (1987)							**↓**					
Nachtman et al. (1988)								↑				

Bold font indicates Tier I studies. The direction of change that is considered adverse for each biomarker is shown at the top of the table. Results are shown with regard to the observed direction of change in each study. Bold arrows indicate a statistically significant effect. A dash represents no change in biomarker level. More than one arrow indicates results at different time points or different conditions. For experimental animal studies, results are shown for the highest exposure level examined.

*Robust against at least one co-pollutant adjustment.

†For this study, the two arrows represent non-asthmatics and asthmatics, respectively.

‡Authors reported that there were no statistically significant changes; measurements not provided.

The Tier II studies of ozone and white blood cell count were cross-sectional analyses. Two large-scale studies in the US reported non-significant decreases in white blood cell counts associated with increases in ambient ozone concentrations (Liao et al. 2005, Schwartz 2001). A cross-sectional study in Israel reported that increased ozone concentrations were associated with increases in white blood cell counts in women at most lag times examined, but with decreases in men; however, none of these changes were statistically significant (Steinvil et al. 2008). Because all of the Tier II studies examining white blood cell counts were cross-sectional, their use for the evaluation of a causal relationship between ozone exposure and changes in white blood cell counts is limited.

IL-6. Only two studies evaluated IL-6; we categorized one as Tier I and the other as Tier II. The Tier I study, a longitudinal analysis of a non-smoking elderly population in the US with a history of coronary heart disease (CHD), evaluated the effects of short-term ambient ozone exposure on circulating IL-6 levels (Delfino et al. 2010). Using single-pollutant models, the authors reported that an increase of 16.1 ppb in 5-day average ozone concentrations (at a lag of 0–4 days) was associated with a non-significant decrease in IL-6 levels. No other lag times were examined.

The Tier II study, a longitudinal analysis of adult volunteers in Canada, examined associations between ozone concentrations and IL-6 levels at various lag times (Thompson et al. 2010). The authors reported that increased ozone concentrations were associated with moderately increased IL-6 levels that were statistically significant for ozone exposure from a lag of 0-days to a lag of 0–5 days, but not at a lag of 0–6 days. Stratified analyses by season showed that increased ozone concentrations at a lag of 0–1 day were associated with increases in IL-6 levels for all seasons except winter, with results for spring and summer being statistically significant. In winter, increased ozone concentrations were associated with a non-significant decrease in IL-6 levels. This study was small, with only 45 participants, and used only single-pollutant models. In addition, the reported changes may not be clinically relevant, as they were moderate increases, and the range of IL-6 levels among participants (0–2.67 pg/mL) was well within the range of normal reference values (0.015–10.1 pg/mL; Ridker et al. 2000).

Other biomarkers of inflammation. Several other biomarkers of inflammation were evaluated in one study each. Bind et al. (2012) examined associations between short-term ozone exposure and levels of ICAM-1 and VCAM-1 in a

study in older men in the US. The authors reported that an IQR increase in 24-h average ozone concentration was associated with a significant increase of 2.3% in ICAM-1 levels at a lag of 0 days. At a lag of 0–2 days, ICAM-1 levels were increased by 1.4%, but not significantly. The clinical relevance of these small increases is unclear, and the mean ICAM-1 level in this population (311.2 ng/mL; standard deviation [SD] of 79.0) was within the range of reported reference values (328 ± 77.4 [SD] ng/mL; Spronk et al. 1994). Increased ozone concentrations were associated with decreases in ICAM-1 levels when longer lags were considered; significant decreases of 4.4% and 4.7% in ICAM-1 levels were observed at lags of 0–20 days and 0–27 days, respectively. Increased ozone concentrations were also associated with decreases in VCAM-1 levels at all lag times examined, except for a lag of 0 days, when a non-significant increase of 2.0% was observed. The authors reported statistically significant decreases in VCAM-1 levels of 7.4%, 8.5%, and 8.7% at lags of 0–13, 0–20, and 0–27 days, respectively. Because the authors used only single-pollutant models, they did not account for potential confounding by co-pollutants.

Another Tier I, longitudinal study by Zhang et al. (2013) examined whether changes in ozone concentrations affect lymphocyte and neutrophil counts in young, healthy, non-smoking participants in China. The authors used bi-pollutant models and reported that a 25.4-ppb increase in 24-h average ozone concentrations at a lag of 0 days was associated with small increases (1.2–3.5%) in lymphocyte count with adjustment for a second co-pollutant, but the changes were not statistically significant except when adjusting for SO_2. In contrast, the same increase in ozone exposure was associated with non-significant decreases in neutrophil count with adjustment for a second co-pollutant.

A Tier II, cross-sectional study in healthy individuals in Italy (Baccarelli et al. 2007a) reported that a 21.4-ppb increase in 24-h average ozone concentrations at a lag of 0 days was associated with a statistically significant increase of 6.7% in fasting tHcy levels. The increase was lower (4.5%) and not statistically significant at a lag of 0–6 days. When post-methionine-load tHcy levels were examined, increased ozone exposure was associated with non-significant changes of 3.6% and − 0.7% at a lag of 0 days and a lag of 0–7 days, respectively. The clinical relevance of the reported increases in tHcy are unclear, as they are small changes, and the mean tHcy level in this population was 9.0 μmol/L (95% CI: 8.8–9.2), which is within the reference range of < 15 μmol/L (Zhang et al. 2014).

Summary. Overall, the findings from the epidemiology studies of ozone and inflammatory biomarkers indicate a lack of consistency in the magnitude, direction, and lag times of the reported changes for the same biomarkers across studies. If ozone induces systemic inflammation, the levels of each of the biomarkers discussed above should have increased in response to increasing ozone exposures. CRP levels were significantly increased at lags of 0–1 day and 0–2 days in one Tier I study (Chuang et al. 2007), and at a lag of 0 days in another (Bind et al. 2012), but were non-significantly increased or decreased at similar lag times in other Tier I and Tier II studies. White blood cell counts were non-significantly decreased at a lag of 0 days and a lag of 1 day in Tier I and Tier II studies, but were non-significantly increased at longer lag times in single-pollutant models, and were significantly increased at a lag of 5 days in bi-pollutant models with NO_2 and SO_2. Levels of IL-6 were non-significantly decreased in a Tier I study at a lag of 0–4 days, but were significantly increased in a Tier II study for the same lag time. The clinical relevance of the reported increases in these biomarkers is unclear, and their levels did not exceed the normal reference ranges in any of the study populations.

Other inflammatory biomarkers were examined in only 1 study each, providing limited evidence for associations. The results indicated few statistically significant increases, all of small magnitude, and all values were within normal reference ranges. In a Tier I study, the markers ICAM-1 and VCAM-1 were increased at a lag of 0 days (though non-significantly for VCAM-1), and significantly decreased when 21- and 28-day moving averages were used as the exposure metric. Another Tier I study reported non-significant increases in lymphocyte counts but non-significant decreases in neutrophil counts in bi-pollutant models at a lag of 0 days. The level of tHcy was significantly increased at a lag of 0 days but not at a lag of 0–6 days in a Tier II study.

Although we consider Tier I studies to be of relatively higher quality than Tier II studies, methodological limitations in the Tier I studies of ozone and inflammatory biomarkers were still present, including potential selection bias, exposure measurement error, and residual and unmeasured confounding. Most of the reported changes were small and not statistically significant, and were sometimes in the opposite direction expected for an increase in systemic inflammation. Given the small magnitude and inconsistent direction of the changes (particularly at the same or similar lag times across studies), as well as the methodological limitations of the studies, the associations are most likely due to bias, confounding, or chance.

Controlled human exposure studies

Seven different inflammatory biomarkers were measured in controlled human exposure studies that evaluated the effects of short-term exposure to 120 or 300 ppb ozone on inflammation (IL-6, CRP, neutrophil count, white blood cell count, TNF-α, IL-8, and IL-1). All of these biomarkers are proinflammatory and should increase in response to ozone exposure, if ozone induces systemic inflammation. The results of these studies are described below and have been summarized in Table 5; detailed study results can be found in Supplementary Table 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371.

Circulating levels of CRP and neutrophils were evaluated in the Tier I study by Brook et al. (2009) and the Tier II study by Devlin et al. (2012). In both studies, CRP levels increased after exposure to ozone; however, the change was only statistically significant 18 h after exposure (but not immediately after exposure) in the study by Devlin et al. (2012). The CRP levels after ozone exposure in the study by Devlin et al. (2012) were < 1 mg/L; thus, the reported increases in this biomarker may not be clinically relevant (as noted above). Neutrophil levels were not significantly changed after ozone exposure in either study. Although the levels were slightly increased immediately after exposure to 300 ppb ozone in the study by Devlin et al. (2012), they were slightly decreased compared to FA controls when measured 18 h after ozone exposure ceased. Brook et al. (2009) also measured white blood cell levels and reported that they were slightly, but not significantly, increased immediately after exposure to 120 ppb ozone and virtually unchanged 24 h after exposure.

Levels of IL-6 were investigated in two studies. Devlin et al. (2012) reported that IL-6 levels were non-significantly increased at both time points after ozone exposure compared to FA exposure. In the Tier I study by Urch et al. (2010), the authors did not provide numerical results, but stated that IL-6 levels were not significantly different after both asthmatics and non-asthmatics were exposed to ozone for 2 h at a concentration of 120 ppb.

TNF-α levels were unchanged after ozone exposure in the Tier I study by Brook et al. (2009), but were non-significantly increased 18 h after exposure in the Tier II study by Devlin et al. (2012). In the Tier I study by Urch et al. (2010), the authors stated that there were no statistically significant changes in this marker after ozone exposure.

Levels of IL-8, an inflammatory chemokine, were significantly increased immediately following ozone exposure, in the study by Devlin et al. (2012). While the levels remained elevated 18 h after exposure, the difference compared to controls was not statistically significant. At both time points after exposure, mean IL-8 levels were well within the reference range of 0.25–3.8 pg/mL (Berrahmoune et al. 2006). In the same study, IL-1 levels were significantly increased 18 h after (but not immediately after) ozone exposure, but were still within the reference range of IL-1 values (0.1–0.41 pg/mL) for healthy adults reported elsewhere (Cigni et al. 2014).

Overall, the controlled human exposure studies of inflammatory biomarkers did not report consistent changes indicative of systemic inflammation in response to ozone exposure (e.g., increased neutrophils, IL-6). Biomarker levels were generally increased, but few to a degree that reached statistical significance, although this may be attributable to the small sample sizes in these studies. Exposure levels in all studies were well above ambient ozone concentrations. Participants in the study by Devlin et al. (2012) were exposed to ozone levels that were more than twice as high as the other studies, and they exercised at high multiples of normal breathing levels during exposure, resulting in a much larger dose of ozone delivered to the airways (Hatch et al. 2013). The clinical relevance of the findings of these studies is unclear, and the increased biomarker levels were all within normal reference ranges after ozone exposure.

Experimental animal studies

Seven different inflammatory biomarkers were measured in the experimental animal studies of ozone (TNF-α, IL-10, IL-6, CRP, white blood cell count, neutrophil count, and lymphocyte count). All of these biomarkers, except IL-10, should increase in response to ozone exposure if ozone induces systemic inflammation. The results of these studies are described below and have been summarized in Table 5; detailed study results can be found in Supplementary Table 3 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371.

Levels of TNF-α were examined in three Tier I studies (Perepu et al. 2010, 2012, Sethi et al. 2012). These studies reported statistically significant increases in TNF-α levels in heart tissue from rats exposed to 800 ppb ozone for 28 days. The study by Perepu et al. (2010) was an *ex vivo* study in which the authors induced ischemia and reperfusion in isolated hearts from ozone-exposed or unexposed rats before biomarker levels were measured. The relevance of such measurements to humans is unclear.

Circulating levels of CRP were non-significantly increased in rats exposed to 810 ppb ozone for 21 days, in the Tier I study by Wang et al. (2013), and 500 ppb ozone for 28 days, in the Tier I study by Jakubowski et al. (2004). By contrast, circulating levels of IL-6 were non-significantly decreased in the study by Wang et al. (2013).

In the Tier I study by Kodavanti et al. (2011), the authors reported non-significant decreases in white blood cell and lymphocyte counts in rats exposed to 500 ppb ozone, and non-significant increases in both of these biomarkers in rats exposed to 1,000 ppb ozone for 2 days. Lymphocyte counts were also examined in two Tier II studies. Bobb and Fairchild (1967) and Fujimaki et al. (1987) reported decreased lymphocyte counts in rats and mice, respectively, after ozone exposure; this change is in the opposite direction expected for an increase in systemic inflammation. In contrast, the Tier II studies by Bobb and Fairchild (1967) and Nachtman et al. (1988) both reported non-significantly increased neutrophil counts in rats after ozone exposure.

Levels of the anti-inflammatory marker IL-10 were significantly decreased in isolated heart tissue after 28 days of exposure to 800 ppb ozone, compared to controls, in the three Tier I studies in which this biomarker was measured (Perepu et al. 2010, 2012, Sethi et al. 2012). As with the findings for TNF-α, it is unclear if the *ex vivo* study by Perepu et al. (2010) is relevant to humans.

Overall, the Tier I experimental animal studies of inflammatory biomarkers reported statistically significant changes in levels of TNF-α and IL-10 that are in the expected direction, if ozone exposure is associated with an increase in systemic inflammation, but these changes were observed in normal or ischemic isolated heart tissue only after very high exposures to ozone. Results for other inflammatory biomarkers in Tier I and Tier II studies were not consistent with respect to the direction of change across studies and were not statistically significant. Levels of white blood cells and lymphocytes were non-significantly decreased in some studies and increased in others, limiting any conclusions regarding these biomarkers. Ozone exposures across the Tier I and Tier II experimental animal studies ranged from 500 to 12,500 ppb, so the relevance of these studies to humans exposed to ambient levels of ozone is unclear. Species differences, such as the fact that rodents breathe only through the nose and have different nasal structures compared to humans, may limit the extrapolation of results in rodents to humans. In addition, rodents have a higher ventilation rate, a higher body surface area/volume ratio, and breathe more air; thus, it is expected that because of these factors, the internal dose of inhaled ozone would be increased (Hatch et al. 2013). Although rodents absorb a smaller fraction of inhaled ozone than humans because of anatomical differences (Miller 1995, Perepu et al. 2010), the high ozone concentrations used in the experimental animal studies may still limit the relevance of the results to humans.

Biomarkers of oxidative stress

Oxidative stress results when the formation of free radicals is unbalanced in proportion to protective antioxidants (Stoner et al. 2013). Several biomarkers of oxidative stress have been examined as biomarkers for CVD, including the oxidative DNA adduct 8-hydroxy-2′-deoxyguanosine (8-OHdG), 8-iso-prostaglandins-$F_{2\alpha}$ (8-iso-PGF), and malondialdehyde (MDA),

which is an indicator of lipid peroxidation (Chuang et al. 2007, Chen et al. 2007, Perepu et al. 2010). In addition, salicylate hydroxylation has been used as a biomarker of hydroxyl radical production. Hydroxyl radical concentrations can be estimated by measuring levels of 2,3-dehydroxybenzoic acid (2,3-DHBA), a metabolite of salicylate (a hydrolyzed form of acetylsalicylic acid, or aspirin) that is produced only when the hydroxyl radical is present (Liu et al. 1997).

The antioxidant enzyme superoxide dismutase (SOD) and the ferric reducing ability of plasma (FRAP) (an antioxidant capacity marker) have also been used as biomarkers for CVD. The levels of both decrease in conditions of increased oxidative stress (Stoner et al. 2013, Chen et al. 2007). Reduced levels of SOD have been reported in patients with stable CAD, MI, and sudden cardiac death (Stoner et al. 2013).

The results of studies that evaluated the effects of short-term ozone exposure on biomarkers of oxidative stress are shown in Table 6.

Epidemiology studies

Two biomarkers of oxidative stress, 8-OHdG and 8-iso-PGF, have been evaluated in epidemiology studies of short-term ozone exposure. Both biomarkers should increase in response to ozone exposure, if ozone induces oxidative stress. The results of these studies are described below and have been summarized in Table 6; detailed study results can be found in Supplementary Table 4 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371.

Three Tier I studies assessed the association between ambient ozone concentrations and 8-OHdG levels, and reported inconsistent findings. A longitudinal analysis in healthy young adults in Taiwan reported that an increase of 17.9 ppb in 24-h average ozone concentrations at a lag of 0 days was associated with a statistically significant 2.2% increase in circulating 8-OHdG levels (Chuang et al. 2007), whereas this biomarker was non-significantly decreased at lags of 0–1 and 0–2 days. Adjustment for sulfate (SO_4^{2-}) yielded a non-significant increase of 1.7% in 8-OHdG levels for each 12-ppb increase in ozone concentrations (at a lag of 0–2 days). Another longitudinal analysis in healthy young adults in China reported that a 25.4-ppb increase in 24-h average ozone concentrations was associated with non-significant decreases in urinary 8-OHdG levels for most single-day lags up to a lag of 2 days, and a statistically significant decrease of 30.6% at a lag of 5 days (Zhang et al. 2013). Using bi-pollutant models, the authors reported that increased ozone exposure at a lag of 5 days was associated with decreases in 8-OHdG levels ranging from 22.0% to 37.1%, with adjustment for a second co-pollutant. The decreases were statistically significant after adjustment for CO, NO_2, SO_2, and sulfate, but were attenuated and not significant after adjustment for PM, EC, and OC, indicating potential confounding by these co-pollutants. Finally, a cross-sectional analysis in older men in the US reported that increases in 1-h maximum ozone concentrations were associated with non-significantly increased urinary 8-OHdG levels at a lag of 0 days, and at lags of 0–6 days and 0–13 days (Ren et al. 2011). At a lag of 0–20 days, the increase in 8-OHdG was 47.7% and statistically significant. However, confounding by co-pollutants was not accounted for in the analysis.

One Tier I study examined associations between ozone and 8-iso-PGF levels. Chen et al. (2007) reported that 8-h maximum ozone concentrations were positively associated with 8-iso-PGF levels at lags of 0–13 and 0–29 days in a cross-sectional analysis of healthy young adults in the US. The effect estimates were small in magnitude and not statistically significant, and the cross-sectional study design limits its use for evaluating a causal relationship between ozone exposure and changes in 8-iso-PGF levels.

Overall, the results from the epidemiology studies of oxidative stress biomarkers are inconsistent with regard to the direction of the effect, both within and across studies. Some studies reported statistically significant increases in 8-OHdG levels with some exposure metrics and lag times, consistent with an increase in oxidative stress, whereas others reported decreases (both significant and non-significant) at similar lag times, even after adjustment for co-pollutants. This inconsistency increases the likelihood that the statistically significant findings are due to chance, bias, and/or confounding.

Controlled human exposure studies

Six different biomarkers of oxidative stress were investigated in controlled human exposure studies that evaluated ozone concentrations ranging from 80 to 500 ppb (2,3-DHBA, the 2,3-DHBA/2,5-DHBA ratio, 8-iso-PGF, FRAP, 8-OHdG adducts, and MDA). All of these biomarkers, with the exception of FRAP, should increase in response to ozone exposure if ozone induces oxidative stress. The results of these studies are described below and have been summarized in Table 6; detailed study results can be found in Supplementary Table 5 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371.

Salicylate hydroxylation was evaluated in two Tier I studies (Liu et al. 1997, 1999). In both studies, participants were given aspirin orally 30 min before their ozone exposure began, and salicylate hydroxylation was determined after exposure by measuring plasma 2,3-DHBA levels. Liu et al. (1997) reported that 2,3-DHBA was significantly increased after a 2-h exposure to both 120 and 400 ppb ozone. The authors also measured the ratio of 2,3-DHBA to its isomer 2,5-DHBA, which is formed from salicylate *via* endogenous enzymatic metabolism rather than by hydroxyl radical. They stated that this ratio should remove some of the variation from potential day-to-day differences in salicylate pharmacokinetics, exercise, diet, and exposure to other oxidants, as the variation in basal salicylate metabolism generally affects both isomers in the same direction. Liu et al. (1997) reported that the 2,3-DHBA/2,5-DHBA ratio was significantly increased (151% change) after exposure to 400 ppb ozone, but not to 120 ppb ozone.

Liu et al. (1999) reported that 2,3-DHBA levels were not significantly increased compared to FA controls immediately after a 2-h exposure to 120 ppb ozone, but were significantly increased 1.5 h after ozone exposure. The clinical relevance of this biomarker remains unclear, as it has not been evaluated thoroughly in the literature for its relevance to CVD endpoints.

The remaining biomarkers of oxidative stress were only evaluated in one Tier II study each. Chen et al. (2007) measured 8-iso-PGF after exposure to 200 ppb ozone for 4 h. Plasma levels of 8-iso-PGF were non-significantly increased 4 h after exposure, but were nearly back to pre-exposure levels

Table 6. Results for biomarkers of oxidative stress.

Studies	2,3-DHBA	2-3,DHBA/ 2,5-DHBA	8-iso-PGF	8-OHdG	FRAP	MDA	SOD
Direction of adverse change	↑	↑	↑	↑	↓	↑	↓
Epidemiology							
Chen et al. (2007)			↑				
Chuang et al. (2007)				↑↓			
Zhang et al. (2013)				↑↓*			
Ren et al. (2011)				↑			
Controlled human exposure							
Liu et al. (1999)	↑↑						
Liu et al. (1997)	↑	↑					
Chen et al. (2007)			↑↑		↓↓		
Bergamischi et al. (2001)†				↑↑			
Buckley et al. (1975)						↑	
Experimental animal							
Liu et al. (1996)	↑						
Chuang et al. (2009)			↑				↓
Chhabra et al. (2010)							↓
Cretu et al. (2010)						↑	
Perepu et al. (2010)						↑	↓
Martinez-Campos et al. (2012)			↑			↑	↓
Perepu et al. (2012)							↓
Sethi et al. (2012)							↓
Kadiiska et al. (2013)			↑↓			↑↓	
Wang et al. (2013)						↑	↓
Feng et al. (2001)				↑		↑	

Bold font indicates Tier I studies. The direction of change that is considered adverse for each biomarker is shown at the top of the table. Results are shown with regard to the observed direction of change in each study. Bold arrows indicate a statistically significant effect. More than one arrow indicates results at different time points or different conditions. For experimental animal studies, results are shown for the highest exposure level examined.

*Robust against at least one co-pollutant adjustment.

†For this study, the two arrows represent participants with risk-related polymorphisms versus those without these polymorphisms.

18 h post-exposure. Chen et al. (2007) also measured antioxidant capacity in the FRAP assay and reported a non-significant decrease in FRAP 4 h post-exposure, but FRAP returned to near pre-exposure levels 18 h after exposure.

Buckley et al. (1975) measured MDA levels in the serum of participants after 2.75 h of exposure to 500 ppb ozone. MDA levels were significantly increased by 85% compared to FA controls, but the clinical relevance of this change is unclear, as the post-exposure levels were low (mean of 0.0018 µmol/L) compared to the reported ranges of serum MDA levels in healthy control populations in other studies (e.g., 0.21–1.49 µmol/L, as reported by Bhutia et al. 2011, or 0.78–1.51 µmol/L, as reported by Lorente et al. 2013).

Bergamaschi et al. (2001) measured 8-OHdG levels in peripheral leukocytes from healthy non-smokers exposed to 80–103 ppb ozone in outdoor air in a semi-experimental study design rather than a controlled exposure scenario. They reported a significant increase in 8-OHdG adducts immediately after ozone exposure in participants with NAD(P)H:quinone oxidoreductase (*NQO1*) wild-type alleles and glutathione-S-transferase µ-1 (*GSTM1*) null polymorphisms; the number of adducts was not significantly increased in participants with *GSTM1*-positive and *NQO1*- null or heterozygous genotypes.

Overall, the Tier I and Tier II controlled human exposure studies reported relatively consistent changes indicative of an increase in oxidative stress at time points within a few hours after exposure to ozone at levels well above ambient concentrations. In a semi-experimental design with lower ozone concentrations (80–103 ppb), significant increases in 8-OHdG levels were only observed in participants with specific genotypes. The results remain limited, however, as all but one of these biomarkers were evaluated in only one Tier II study each, so further studies are needed to validate the findings.

Experimental animal studies

Five different biomarkers of oxidative stress were evaluated in the experimental animal studies included in our analysis (SOD, 2,3-DHBA, MDA, 8-OHdG, and 8-iso-PGF). All of these biomarkers, with the exception of SOD, should increase in response to ozone exposure if ozone induces oxidative stress. The results of these studies are described below and have been summarized in Table 6; detailed study results can be found in Supplementary Table 6 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371.

Seven Tier I studies evaluated the antioxidant enzyme SOD, and all reported decreased SOD activity after ozone exposure, with most results being statistically significant compared to those of control animals. Most of these studies evaluated changes in SOD in mice (Chuang et al. 2009) or rats (Perepu et al. 2010,

2012, Martinez-Campos et al. 2012, Sethi et al. 2012, Wang et al. 2013) for ozone exposures in the range of 485–800 ppb, except for the study by Chhabra et al. (2010), who assessed SOD changes in guinea pigs after 4 weeks of exposure to 120 ppb ozone. SOD was measured in different biological media across studies. Chhabra et al. (2010) and Martinez-Campos et al. (2012) measured SOD in blood and plasma, respectively, while the other studies examined SOD in normal or ischemic-injured rat heart tissue (Perepu et al. 2010, 2012, Sethi et al. 2012, Wang et al. 2013) or mouse aortic tissue (Chuang et al. 2009).

Levels of 8-iso-PGF were examined in three Tier I studies, and were significantly increased in mouse aortic tissue (Chuang et al. 2009) and rat arterial blood (Martinez-Campos et al. 2012) after exposure to 500 ppb ozone for 5 days or 14 days, respectively. Kadiiska et al. (2013) reported both slight increases and decreases in plasma and urine levels of 8-iso-PGF in rats exposed to 2,000 or 5,000 ppb ozone for 2 h, depending on the post-exposure time point at which it was measured. The authors concluded that there was no overall significant change in the levels of this biomarker of lipid peroxidation.

Five Tier I studies and one Tier II study examined changes in MDA levels in rats after ozone exposure. Circulating MDA levels were significantly increased in rats exposed to 500 or 800 ppb ozone for 14 or 28 days in three Tier I studies (Cretu et al. 2010, Perepu et al. 2010, Martinez-Campos et al. 2012). Circulating MDA levels were effectively unchanged (i.e., both slightly increased and decreased across time points of measurement) in rats after exposure to 2,000 or 5,000 ppb ozone for 2 h in one other Tier I study (Kadiiska et al. 2013). Kadiiska et al. (2013) also measured urinary levels of MDA and reported similar, inconsistent results. Another Tier I study reported a slight, but not significant, increase in MDA in rat heart tissue after exposure to 810 ppb ozone for 21 days (Wang et al. 2013). In the Tier II study by Feng et al. (2001), MDA levels in mouse heart tissue were significantly increased after exposure to 600 ppb ozone for 20 days, but not after exposure for 10 days. The authors also examined exposure durations of five and 15 days, but did not report the results.

Levels of 2,3-DHBA and 8-OHdG adducts were examined in one study each. The Tier I study by Liu et al. (1996) reported statistically significant increases in 2,3-DHBA levels in rat plasma after exposure to 1,000 or 2,000 ppb ozone. The Tier II study by Feng et al. (2001) also reported significant increases in 8-OHdG adducts in urine from mice exposed to 600 ppb ozone for 15 days, but the authors did not report the results when the exposure duration was 5, 10, or 20 days. The study by Feng et al. (2001) had several methodological limitations, including the use of inappropriate statistical methods (see Table 4); thus, this single study does not provide reliable evidence for a significant effect of ozone on 8-OHdG adduct levels.

Overall, the Tier I experimental animal studies of biomarkers of oxidative stress reported relatively consistent increases in the levels of 8-iso-PGF and MDA, as well as consistent decreases in SOD activity across species and tissues after exposure of rodents to high concentrations of ozone. These changes are in the expected direction of an adverse effect of ozone on inducing oxidative stress in the CV system. Ozone exposures in all but one of the studies ranged from 500 to 5,000 ppb; thus, as noted previously, the relevance of these results to humans exposed to ambient ozone concentrations is unclear.

Biomarkers of coagulation and arterial vasoreactivity

Many biomarkers associated with coagulation have been studied as risk factors for CVD. These include procoagulant proteins such as fibrinogen, thrombin, von Willebrand factor (vWF), plasminogen activator inhibitor-1 (PAI-1), and tissue factor (TF). In the coagulation cascade, fibrinogen is converted by thrombin into insoluble strands of fibrin. Blood clots are formed from cross-linked fibrin strands and an aggregation of platelets. Fibrinogen is also a marker of inflammatory processes, as it is an acute-phase response protein that is synthesized in the liver upon induction by cytokines such as IL-6.

Biomarkers of anticoagulation such as thrombomodulin, plasminogen, tissue-type plasminogen activator (tPA), and D-dimer have also been examined as biomarkers of CVD risk. Thrombomodulin decreases blood coagulation by converting thrombin from a procoagulant enzyme to an anticoagulant enzyme (Dittman and Majerus 1990). The protease tPA catalyzes the conversion of plasminogen to plasmin, an enzyme that breaks down blood clots (Devlin et al. 2012).

Because high blood pressure is a classic risk factor for CVD (Lloyd-Jones et al. 2010, Folsom 2013), several markers of vasoreactivity (i.e., changing diameter of blood vessels) have been studied as biomarkers of CVD. Atrial natriuretic factor (ANF), also known as atrial natriuretic peptide (ANP), is a vasodilator that increases in the circulation in response to elevated blood pressure (Vesely et al. 1994a). Endothelin-1 (ET-1) is a vasoactive peptide that contributes to the maintenance of vascular tone and is associated with increased blood pressure (Wang et al. 2013). Although it is a marker of angiogenesis rather than vasoreactivity, vascular endothelial growth factor (VEGF) can adversely affect vascular homeostasis if overexpressed (Wang et al. 2013).

The results of studies that evaluated the effects of short-term ozone exposure on biomarkers of coagulation and vasoreactivity are shown in Table 7.

Epidemiology studies

Nine biomarkers of coagulation have been evaluated in ozone epidemiology studies (fibrinogen, vWF, platelet aggregation, PAI-1, tPA, endogenous thrombin potential [ETP], thrombin peak height [TPH], TF, and thrombomodulin). All of these biomarkers, with the exception of tPA and thrombomodulin, should increase in response to ozone exposure if ozone induces adverse effects on coagulation. The results of these studies are described below, and have been summarized in Table 7; detailed study results can be found in Supplementary Table 7 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371.

Fibrinogen. Six Tier I studies examined the association between short-term ozone exposure and fibrinogen levels. Their findings were mixed. A longitudinal analysis in healthy young adults in Taiwan reported that IQR increases in ambient ozone concentrations were associated with significant increases in fibrinogen levels of 4.8–6.9% for each lag time examined (Chuang et al. 2007). The effect estimate for ozone was attenuated and did not maintain statistical significance when sulfate was adjusted for in the analysis. A longitudinal

analysis in healthy individuals in the Netherlands reported that a 21-ppb increase in 24-h average ozone concentration was associated with small decreases in fibrinogen levels at lags of 1 and 2 days, and a small increase at a lag of 3 days (Rudez et al. 2009). None of these changes were statistically significant. A longitudinal analysis in older men in the US reported both increases and decreases in fibrinogen associated with increased ozone concentrations at different lags (Bind et al. 2012). There was no pattern among the changes, which were very close to null and not statistically significant. Longitudinal analyses in healthy, young, non-smoking adults in China reported that an increase of 25.4 ppb in 24-h average ozone concentrations was associated with small, non-significant decreases in fibrinogen levels at a lag of 0 to a lag of 7 days (Rich et al. 2012, Zhang et al. 2013). A large-scale, cross-sectional study in the United Kingdom reported that an increase of 23.45 ppb in 8-h maximum ozone concentrations was associated with small increases in fibrinogen levels at lags of 0 or 1 day, and small decreases at lags of 2 or 3 days (Pekkanen et al. 2000). Stratified analyses by smoking status, sex, or season did not show any particular pattern in the changes in fibrinogen levels. The effect estimates for ozone at various lags were all very small (ranging from a decrease of 1.34% to an increase of 3.07%) and not statistically significant.

Five Tier II studies investigated changes in fibrinogen levels associated with ozone exposure. A longitudinal analysis in adult volunteers in Canada reported small increases in fibrinogen levels associated with increased ozone concentrations at all lag times examined, but the changes were not statistically significant (Thompson et al. 2010). Of two large-scale, cross-sectional studies in the US, Schwartz (2001) reported a non-significant, positive association between ozone and fibrinogen levels at a lag of 0 days, and Liao et al. (2005) reported a significant nonlinear effect of ozone on fibrinogen at a lag of 1 day. Both studies evaluated ozone only in single-pollutant models. A cross-sectional study in Israel reported that an increase of 15 ppb in ozone concentrations was associated with a significant 4.2% increase in fibrinogen levels in men at a lag of 4 days (but at no other lag times, as shown in Supplementary Table 7 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371) without adjustment for co-pollutants and a 6.9% decrease in fibrinogen levels in men at a lag of 4 days with adjustment for PM_{10}, SO_2, and NO_2 (Steinvil et al. 2008). There were no associations in women at any of the lag times examined (a lag of 0 to a lag of 7 days). A cross-sectional analysis of healthy individuals in Italy reported that an increase in ozone concentrations of one SD was associated with significant decreases in fibrinogen levels at lags of 0–6 and 0–29 days, but not at a lag of 0 days, from single-pollutant models (Baccarelli et al. 2007b).

Together, the epidemiology studies of fibrinogen reported changes that were inconsistent in direction across studies. Only one of six Tier I studies (Chuang et al. 2007) and two of five Tier II studies (Schwartz 2001, Steinvil et al. 2008) reported statistically significant increases in fibrinogen levels associated with ozone exposure in single-pollutant models. These associations were not observed in bi- or multi-pollutant models, indicating that the results could be confounded by co-pollutants. In addition, the clinical relevance of the increases is unclear, given their small magnitude, and participants in all three studies had fibrinogen levels that were within the normal range of 150 to 450 mg/dL (Kamath and Lip 2003).

vWF. Two Tier I studies of the same population reported a decrease in vWF levels with increased ozone exposure, a change that is in the opposite direction expected for an adverse effect of ozone on coagulation. Rich et al. (2012) conducted a longitudinal analysis in young, healthy, non-smoking adults in China and reported that an increase of 25.4 ppb in 24-h average ozone concentrations was associated with a significant decrease in vWF levels of 19.2% at a lag of 0 days. The changes in vWF were attenuated at longer lags of up to 6 days and lost statistical significance. A re-analysis of this study by Zhang et al. (2013) showed that the decreases in vWF associated with increased ozone concentrations were robust with adjustment for a second co-pollutant, including PM, NO_2, CO, SO_2, EC, OC, or sulfate. The vWF levels in the study participants were within the reference range, reported as 50–200% of the mean vWF concentration in a standard human plasma pool (Sadler 2003).

Changes in vWF levels were also examined in a Tier II study. Liao et al. (2005) conducted a cross-sectional analysis in the US and reported that the effect of ozone on vWF was nonlinear, but not statistically significant.

Platelet aggregation. Two Tier I studies evaluated the association between short-term ozone exposure and platelet aggregation and reported inconsistent results. In a longitudinal analysis in healthy individuals in the Netherlands, Rudez et al. (2009) reported that an increase of 21 ppb in 24-h average ozone concentrations was associated with a small increase in maximum platelet aggregation in plasma at a lag of 0 days, and small decreases in longer single-day lags. The changes were not statistically significant, and the overall effect at lags of 0–3 days was a decrease in platelet aggregation of 7.2%. The authors also examined late platelet aggregation, which is residual aggregation measured 6 min after maximum aggregation and represents platelet aggregate stability. Increased 24-h average ozone concentrations were associated with moderate decreases in late platelet aggregation at most lags examined. When 1-h maximum ozone concentration was used as the exposure metric, increased ozone exposure at a lag of 0 days was associated with a significant decrease of 16.4% in late platelet aggregation.

A longitudinal analysis in young, healthy, non-smoking Chinese adults (Zhang et al. 2013) showed that an increase of 25.4 ppb in 24-h average ozone concentrations was associated with significant increases (7.4–13.3%) in platelet aggregation at a lags of 0 and 1 day. The changes attenuated at later lags and lost statistical significance. The significant association between ozone concentrations at a lag of 0 days and platelet aggregation remained robust after adjustment for co-pollutants, with the exception of CO. The clinical relevance of the increases in platelet aggregation in this study is unclear, as the percentage of platelet aggregation in study participants was within the normal range (63–97%; Helena Laboratories 2012).

Other biomarkers of coagulation. Several other biomarkers of coagulation were examined in one study each. Chuang et al. (2007), a Tier I, longitudinal study in healthy young adults in

Table 7. Results for biomarkers of coagulation and vasoreactivity.

Studies	ANF	D-Dimer	Endothelin-1	ETP	Fibrinogen	PAI-1	Plasminogen	Platelet Aggregation	Thrombomodulin	Tissue Factor (TF)	tPA	TPH	VEGF	vWF
Direction of adverse change	↑	↑	↑	↑	↑	↑	↑	↑	↓	↑	↓	↑	↑	↑
Epidemiology														
Pekkanen et al. (2000)					↑↓									
Chuang et al. (2007)					**↑**	**↑***					↑			
Rudez et al. (2009)				↑↓	↑↓			↑**↓**				**↑**		
Bind et al. (2012)					↑↓									
Rich et al. (2012)					↓									↑**↓**
Zhang et al. (2013)					↓			**↑***↓						**↓***
Liao et al. (2005)					↑↓									↑↓
Baccarelli et al. (2007b)					**↓**									
Steinvil et al. (2008)					**↑↓***									
Thompson et al. (2010)					↑									
Poursafa et al. (2011)									**↓**	**↑**				
Schwartz (2001)					↑									
Controlled human exposure														
Devlin et al. (2012)		↑↓				**↓↓**	↑**↓**				↑↑			↑↓
Strak et al. (2013)				↓↑								↑↑		
Gong et al. (1998)	– – †													
Experimental animal														
Vesely et al. (1994a)	**↑**													
Bouthillier et al. (1998)			↓↑											
Sanchez-Gonzalez et al. (2004)			↓											
Thomson et al. (2006)			↑											
Wang et al. (2013)			↓										–	

Bold font indicates Tier I studies. The direction of change that is considered adverse for each biomarker is shown at the top of the table. Results are shown with regard to the observed direction of change in each study. Bold arrows indicate a statistically significant effect. A dash represents no change in biomarker level. More than one arrow indicates results at different time points or different conditions. For experimental animal studies, results are shown for the highest exposure level examined.

*Robust against at least one co-pollutant adjustment.

†Authors reported that there were no statistically significant changes; measurements not provided.

Taiwan, reported that an increase of 12 ppb in 3-day average ozone concentrations was associated with a significant increase of 33% in PAI-1 levels at a lag of 0–2 days. This increase was attenuated to 9.2% with adjustment for sulfate, but maintained statistical significance. However, levels of PAI-1 in study participants were lower than those measured in other normal populations (Mooij et al. 2011), so the clinical relevance of these increases is unclear. Chuang et al. (2007) also reported that IQR increases in ozone concentrations were associated with non-significant increases in tPA levels at a lag of 0 days, and at lags of 0–1 and 0–2 days. As noted above, increased PAI-1 levels indicate procoagulation, whereas increased tPA levels indicate anticoagulation. It is not biologically plausible that ozone would affect both processes simultaneously, so it is likely that the results for one or both of these biomarkers are attributable to bias, confounding, or chance.

Another Tier I, longitudinal study in healthy individuals in the Netherlands investigated whether short-term ambient ozone exposures were associated with thrombin generation by evaluating ETP, TPH, and lag time of thrombin generation (Rudez et al. 2009). ETP and TPH reflect the potential of plasma to generate thrombin, and increases in these markers have been suggested to indicate hypercoagulability (Strak et al. 2013). Rudez et al. (2009) reported that increases in 1-h maximum ozone concentrations at a lag of 0 days were associated with a significant increase of 6.3% in TPH, a non-significant increase of 2.3% in ETP, and a non-significant decrease of 1.2% in the lag time of thrombin generation. When average ozone concentrations were used as the exposure metrics, changes in thrombin generation parameters were small, not significant, and not in a consistent pattern. The results of this study were from single-pollutant models, and thus did not account for confounding by co-pollutants.

A Tier II, cross-sectional study in healthy children and adolescents in Iran by Poursafa et al. (2011) reported that quartiles of 24-h average ozone concentrations were positively associated with the top quartile of TF levels at a lag of 0 days, with a statistically significant linear trend. Similarly, quartiles of ozone concentrations were negatively associated with the top quartile of thrombomodulin levels, with a statistically significant linear trend. Both associations were consistent with an effect of procoagulation, but the analyses did not include adjustment for co-pollutants, temporal, or meteorological factors. Also, the cross-sectional design of the study limits its use for evaluating a causal relationship between ozone exposure and changes in biomarkers of coagulation.

Summary. Overall, there are no consistent findings across studies for the effect of short-term ozone exposure on biomarkers of coagulation. Tier I and Tier II studies reported small effect estimates for fibrinogen that were not always in the same direction, with a few statistically significant increases with unclear clinical relevance. Two Tier I studies suggest ozone may be associated with moderate decreases in vWF levels, but these effects would not be considered adverse, as vWF is a procoagulant protein. Two Tier I studies with similar design and methods reported conflicting findings with regard to the direction of the effects on platelet aggregation, but it is unclear whether these results were clinically relevant or specific to different study populations. The levels of most of the other biomarkers of coagulation examined in one study each were not evaluated in multi-pollutant models, or there were no consistent patterns in the direction of alteration across exposure metrics within studies. The overall inconsistency of results in the epidemiology studies increases the likelihood that the findings are attributable to chance, bias, or confounding.

Controlled human exposure studies

Circulating levels of eight individual biomarkers of coagulation or vasoreactivity were evaluated in controlled human exposure studies that examined ozone concentrations of 7 or 300 ppb: ANF, ETP, TPH, PAI-1, tPA, vWF, plasminogen, and D-dimer. All of these biomarkers, with the exception of tPA and D-dimer, should increase in response to ozone exposure if ozone induces adverse effects on coagulation or vasoactivity. Each biomarker was evaluated in one Tier II study. The results of these studies are described below and have been summarized in Table 7; detailed study results can be found in Supplementary Table 8 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371.

Gong et al. (1998) reported that levels of ANF were not significantly changed in response to 3-h chamber exposures to 300 ppb ozone. The authors also examined several prohormone-ANF peptides, one of which (pro-ANF 1–30) was slightly, but not significantly, decreased after exposure. This change is not in the expected direction of an adverse effect on CVD risk. The authors did not provide the actual ANF measurements, so the absolute changes in these markers after ozone exposure cannot be evaluated.

Strak et al. (2013) measured ETP and TPH in a semi-experimental, crossover study in which participants were exposed to an average of 7 ppb ozone for 5 h at several different outdoor sites in the Netherlands. The authors reported that levels of ETP were slightly decreased 2 h after exposure, but were not significantly different from levels in FA controls. The next morning, ETP levels were increased 14% compared to pre-exposure values, but again were not significantly different from levels in FA controls. TPH levels were increased by 20% and 10% compared to pre-exposure values, 2 h after exposure and the next morning, respectively. These changes were not statistically significant when compared to FA controls.

Devlin et al. (2012) measured five other markers of coagulation (PAI-1, tPA, vWF, plasminogen, and D-dimer) in participants exposed to 300 ppb ozone for 2 h. Levels of PAI-1 were decreased immediately after exposure and remained decreased compared to FA controls 18 h after exposure. While these changes were statistically significant, they are in the opposite direction of what one would expect for an adverse effect on coagulation. Levels of tPA were non-significantly increased both immediately and 18 h after exposure; these changes are also in the opposite direction of an adverse effect. Levels of vWF were increased, albeit not significantly, immediately after ozone exposure; a similar magnitude of increase was also reported after FA exposure. By 18 h post-exposure, vWF levels had decreased to below pre-exposure values. Plasminogen levels were initially increased, but this was not a statistically significant change compared to FA controls. By 18-h post-exposure, plasminogen levels had significantly decreased compared to FA control levels; a decrease in plasminogen levels does not indicate an adverse effect on coagulation. Levels of D-dimer were increased slightly, but not significantly

immediately after exposure when compared to controls. By 18 h post-exposure, levels of D-dimer were lower than pre-exposure and FA levels, although these differences were not significant.

Overall, the controlled human exposure studies examining effects of ozone on biomarkers of coagulation and vasoreactivity reported non-significant changes in biomarker levels that were inconsistent in direction. The few statistically significant changes were observed with high (300 ppb) ozone exposure and all were in the opposite direction of what would be considered an adverse effect of ozone on coagulation. Each of the biomarkers was measured in only one Tier II study, and the methodological limitations of these studies limit their use for assessing associations between ozone exposure and changes in biomarkers of coagulation and vasoreactivity.

Experimental animal studies

Three different biomarkers related to vasoreactivity (ET-1, ANF, and VEGF) were evaluated in the experimental animal studies included in our analysis. All of these biomarkers should increase in response to ozone exposure if ozone induces adverse effects on arterial vasoreactivity. The results of these studies are described below, and have been summarized in Table 7; detailed study results can be found in Supplementary Table 9 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371.

Circulating levels of the vasoconstriction factor ET-1 were examined in four Tier I studies (Bouthillier et al. 1998, Sanchez-Gonzalez et al. 2004, Thomson et al. 2006, Wang et al. 2013). Sanchez-Gonzalez et al. (2004) reported a significant decrease in ET-1 levels in rats after 14 days of exposure to 250 ppb ozone, but the decreases were not significant after a shorter (7 days) or longer (28 days) exposure duration. Wang et al. (2013) reported no changes in ET-1 levels in rats exposed to 810 ppb ozone for 21 days. Two studies reported non-significant increases in ET-1 levels after rats were exposed to 800 ppb ozone for 4 h (Thomson et al. 2006) or 3 days (Bouthillier et al. 1998); the latter study also reported a slight, non-significant decrease in ET-1 with an exposure duration of 1 day.

The other two biomarkers of vasoreactivity were evaluated in a single Tier I study each. Serum levels of VEGF were unchanged in rats in response to repeated exposure to 810 ppb ozone for 21 days (Wang et al. 2013). Circulating and heart tissue levels of ANF and its prohormone peptides were significantly increased in both adult (4–6 months old) and "aged" (24–26 months old) rats after exposure to 500 ppb ozone for 8 h (Vesely et al. 1994a). The same data were also reported by the same group of investigators in other publications (Vesely et al. 1994b,c).

Overall, the only biomarker of vasoreactivity examined in more than one experimental animal study, ET-1, was significantly decreased in rats exposed to 250 ppb ozone for 14 days, but was non-significantly increased or decreased in rats exposed to 800 ppb for various exposure durations, with no pattern of change with increasing duration. There were also no significant effects on ET-1 levels in another study with exposure to 810 ppb ozone for a duration in a similar range. The significant increase in ANF levels after acute exposure to 500 ppb ozone is considered to be an adverse change in relation to vasoreactivity, but this finding has not been replicated in other studies. Given the high ozone exposures in all of these studies, the relevance of the results to humans exposed to ambient ozone concentrations is unclear.

Biomarkers of lipid and glucose metabolism

High circulating levels of total cholesterol and high-density lipoprotein (HDL) cholesterol are classic risk factors for CVD. In addition, high triglyceride levels are implicated as risk factors for CAD and stroke. Other blood lipid biomarkers include lipoprotein-associated phospholipase A2 (Lp-PLA2), an enzyme produced by inflammatory cells within atherosclerotic lesions that converts oxidized LDL in the subendothelial space to oxidized free fatty acids and lysophosphatidylcholine, which trigger an inflammatory cascade. Apolipoproteins are proteins that bind to lipids to form lipoproteins, such as LDL and HDL. Apolipoprotein B (ApoB) is the primary protein of LDLs and has been shown to be a better indicator of atherosclerotic risk than total cholesterol or non-HDL cholesterol (Chuang et al. 2010). Apolipoprotein A1 (ApoA1) is the major protein component of HDL (Walldius and Jungner 2005). Hemoglobin A1c (HbA1c) levels are used by clinicians to monitor the degree of control over glucose metabolism; increases in HbA1c are associated with the development of atherosclerotic plaques (Chuang et al. 2010).

The results of studies that evaluated the effects of short-term ozone exposure on biomarkers of blood lipid and glucose metabolism are shown in Table 8.

Epidemiology studies

Two Tier I epidemiology studies evaluated the association between short-term ozone exposure and biomarkers of lipid and glucose metabolism. A total of seven biomarkers were investigated (Lp-PLA2, ApoB, ApoA1, triglycerides, HbA1C, LDL cholesterol, and HDL cholesterol), but none in more than one study. All of these biomarkers, with the exception of ApoA1 and HDL cholesterol, should increase in response to ozone exposure if ozone induces adverse effects on lipid and glucose metabolism. The results of these studies are described below, and have been summarized in Table 8; detailed study results can be found in Supplementary Table 10 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371.

A longitudinal analysis in MI survivors in Germany using single-pollutant models reported that a 30.95-ppb increase in 8-h average ozone concentrations at a lag of 0 days was associated with a significant 2.32% increase in Lp-PLA2 levels (Bruske et al. 2011). The clinical relevance of this change is unclear, however, because of the small magnitude of the increase and because most study participants had Lp-PLA2 levels within the normal range of <200 ng/mL (Davidson et al. 2011). The changes in Lp-PLA2 levels at later single-day lags were smaller and non-significant increases, and at a lag of 5 days, levels of Lp-PLA2 were non-significantly decreased by 1.32%.

A large-scale, cross-sectional study conducted in Taiwan assessed the effect of short-term ozone exposure on circulating levels of ApoA1, ApoB, blood triglycerides, HDL cholesterol, LDL cholesterol, and HbA1c in the general population (Chuang et al. 2010). An increase of 12.2 ppb in 24-h ozone concentrations was associated with small, non-significant changes that are consistent with increased CVD risk (i.e., decreases in ApoA1 and HDL cholesterol, and increases in ApoB, triglycerides, and LDL cholesterol) at lags of 0 days, 0–2 days, and 0–4 days, although for one lag time examined (a lag of 0–4 days), the non-significant increase in ApoA1 levels is consistent with reduced CVD risk. Increased ambient ozone concentrations were also associated

statistically significant increases (0.05–0.07%) in levels of the glucose metabolism marker (HbA1c) at all lag times examined. While the range of HbA1c levels in this population (3.5–14.7%) exceeds the reference range of < 8% (Leikin and Paloucek 2008), the reported increases were very small, so their clinical relevance is unclear.

Overall, the only biomarkers of blood lipids and glucose metabolism with statistically significant changes associated with increased ozone exposure were Lp-PLA2 and HbA1c. Ozone exposure was associated with small increases in the levels of both biomarkers, so their clinical relevance is unclear. For the other biomarkers, the non-significant changes were small, but in the direction consistent with an increased risk of CVD. All of the biomarkers were only analyzed in single-pollutant models; therefore, confounding by co-pollutants cannot be ruled out.

Controlled human exposure studies

Total cholesterol was the only biomarker of lipid metabolism that was examined in a controlled human exposure study. This biomarker should increase in response to ozone exposure if ozone induces adverse effects on lipid metabolism. The results of this study are described below, and have been summarized in Table 8; detailed study results can be found in Supplementary Table 11 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371.

In the Tier II study by Devlin et al. (2012), cholesterol levels were increased in participants after 2 h of exposure to 300 ppb ozone compared to FA exposure; however, this change was slight (2.2 mg/dL) and not statistically significant. Follow-up cholesterol levels 18 h after exposure were slightly lower than FA control levels at that time point, but this difference was not significant. Without measures of other blood lipids in this study, and in the absence of analyses of blood lipids in other controlled human exposure studies, no conclusions can be made regarding an effect of ozone exposure on this category of biomarkers.

Experimental animal studies

Four biomarkers of lipid metabolism (triglycerides, total cholesterol, and HDL and LDL cholesterol) were evaluated in the experimental animal studies included in our analysis. All of these biomarkers, with the exception of HDL cholesterol, should increase in response to ozone exposure if ozone induces adverse effects on blood lipids, whereas HDL cholesterol levels would be expected to decrease. The results of these studies are described below and have been summarized in Table 8; detailed study results can be found in Supplementary Table 12 to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2015.1031371.

Three studies evaluated changes in total cholesterol levels in response to ozone exposure. The Tier I study by Mole et al. (1985) reported a statistically significant increase in total cholesterol in rats 20 days after exposure to 3,000 ppb ozone for 14 days, but not with lower exposures (1,000 and 1,750 ppb). Mole et al. (1985) also reported a significant increase in HDL cholesterol in rats exposed to 3,000 ppb ozone, a change that is not in the direction consistent with an increased risk of CVD.

The Tier II study by Vaughan et al. (1984) reported a statistically significant increase in total cholesterol as well as LDL cholesterol in guinea pigs, immediately after a 14-day exposure to 1,000 ppb ozone. These effects were ameliorated and not significant when measured 30 days later.

The Tier II study by Takatori (1975) reported both an increase and a decrease in total cholesterol levels in rats, depending on the exposure time point. Rats exposed to 1,100 ppb for 24 h had non-significant increases in cholesterol levels. After a 72-h period of exposure to room air, these rats were exposed to ozone again for another 24 h, resulting in a significant increase in the levels of total cholesterol compared to pre-exposure levels. Exposure to 2,500 ppb ozone for 24 h resulted in a non-significant increase in cholesterol, whereas a second 24-h exposure period to 2,000 ppb ozone 72 h after the first exposure period resulted in a non-significant decrease in cholesterol levels compared to pre-exposure levels.

Two studies examined triglyceride levels in response to ozone exposure. The Tier I study by Mole et al. (1985) reported a non-significant increase in triglyceride levels in rats after exposure to 1,000 ppb ozone for 14 days, and a non-significant decrease in triglyceride levels when exposures were to 1,750 or 3,000 ppb ozone. The Tier II study by Vaughan et al. (1984) reported a non-significant decrease in triglyceride levels in male guinea pigs and a non-significant increase in this biomarker in female guinea pigs immediately

Table 8. Results for biomarkers of lipid and glucose metabolism.

Studies	ApoA1	ApoB	HDL Cholesterol	Hemoglobin A1c	LDL Cholesterol	Lp-PLA2	Total Cholesterol	Triglycerides
Direction of adverse change	↑	↑	↓	↑	↑	↑	↑	↑
Epidemiology								
Chuang et al. (2010)	↑↓	↑	↓	**↑**	↑			↑
Bruske et al. (2011)						**↑↓**		
Controlled human exposure								
Devlin et al. (2012)							↑–	
Experimental animal								
Mole et al. (1985)			**↑**				**↑**	↓
Takatori (1975)							↑↓	
Vaughan et al. (1984)					**↑**		**↑**	↓↑

Bold font indicates Tier I studies. The direction of change that is considered adverse for each biomarker is shown at the top of the table. Results are shown with regard to the observed direction of change in each study. Bold arrows indicate a statistically significant effect. A dash represents no change in biomarker level. More than one arrow indicates results at different time points or different conditions. For experimental animal studies, results are shown for the highest exposure level examined.

after exposure to 1,000 ppb ozone for 14 days. When triglyceride levels were measured 30 days after exposure ended, there was a non-significant increase in males and non-significant decrease in females.

Overall, the experimental animal studies of biomarkers of lipid metabolism reported inconsistent results, both within and across studies. The only Tier I study reported non-significant decreases in triglycerides and significant increases in HDL cholesterol at the highest exposure concentration (3,000 ppb). These effects are not consistent with an increased risk of CVD. By contrast, this study reported significant increases in total cholesterol with exposure to 3,000 ppb ozone. The two Tier II studies reported significant increases in total cholesterol immediately after exposure to ozone concentrations of around 1,000 ppb, but not with higher concentrations. The Tier II study that examined triglyceride levels in guinea pigs reported non-significant decreases in males and increases in females immediately after exposure, and non-significant changes in the opposite direction for each sex, when measured 30 days after exposure ended. All three studies of biomarkers of lipid metabolism used very high concentrations of ozone (i.e., $\geq$ 1,000 ppb) for long exposure durations; thus, the relevance of the results to humans exposed to ambient ozone concentrations is unclear.

Summary

In the epidemiology studies reviewed above, the reported associations (both statistically significant and non-statistically significant) between short-term ozone exposure and changes in atherosclerosis-related biomarker levels in all categories were inconsistent in direction and lag time, both within and across studies. There were very few statistically significant changes in the direction of an adverse effect on the CV system, and most of these were reported from studies that used only single-pollutant models, therefore confounding by co-pollutants cannot be ruled out. In most cases, the changes were small in magnitude and may not be clinically relevant. Most of the epidemiology studies had adequate study size and QA/QC protocols, and most used appropriate statistical models and evaluated multiple lags. Although we classified the majority of epidemiology studies as Tier I because of these strengths, methodological limitations were still present, such as potential selection bias, exposure measurement error, confounding, and lack of adjustment for multiple comparisons. Because of the overall inconsistency of the results, it is unclear whether the statistically significant findings are attributable to at least some of these factors.

In the controlled human exposure studies, the only statistically significant findings for biomarkers of inflammation were reported in one Tier II study at a high exposure (300 ppb). The effects were small in magnitude and may not be clinically relevant. Two Tier I studies reported significantly increased salicylate hydroxylation after exposure to at least 120 ppb ozone, and several other biomarkers of oxidative stress were increased at exposures of at least 200 ppb in Tier II studies. All biomarkers of coagulation, vasoreactivity, and lipid metabolism were evaluated in only one Tier II study each. These studies reported either non-significant changes that were inconsistent in direction, or significant changes that were in the opposite direction of what would be considered an adverse effect on the CV system. While some of these studies had many strengths, such as crossover designs, blinding of exposure status, and adequate statistical and QA/QC methods, most had small study sizes and no power calculations to ensure sufficient power to detect changes in biomarker levels; thus, more studies are needed to validate the findings for these categories of biomarkers.

Although the experimental animal studies reported changes in biomarker levels that were mostly inconsistent in direction after ozone exposure, several biomarkers were consistently changed in the direction expected of an adverse effect on the CV system. Levels of the inflammatory markers TNF-α and IL-10 were significantly altered in rat heart tissues across Tier I studies, and levels of the oxidative stress-related biomarkers 8-iso-PGF, MDA, and SOD were relatively consistently changed across species in Tier I studies. The relevance of these changes to humans exposed to ambient ozone concentrations is unclear, however, given the differences between species and the very high ozone exposures (generally $\geq$ 500 ppb) used in the majority of experimental animal studies.

Integration of evidence across realms

In the preceding sections, we evaluated the reported changes in biomarkers of inflammation, oxidative stress, coagulation/vasoreactivity, and lipid/glucose metabolism in response to ozone exposure in studies within different realms of investigation (epidemiology, controlled human exposure, and experimental animal). Below, we integrate the data across all realms of evidence so that the evaluation of each realm informs the interpretation of the others. We consider several aspects to aid in our judgments regarding the WoE for causal relationships between ozone and adverse changes in levels of atherosclerosis-related biomarkers. These include the Bradford Hill criteria of strength of association, consistency of associations, coherence, biological plausibility, biological gradient (exposure-response), temporality, specificity, and experimental evidence (Hill 1965). The Bradford Hill criteria were developed mainly for the interpretation of epidemiology results, but they are applicable to other study types, so we use them for evaluating studies from different realms. We also consider confounding and bias among the studies, as well as the potential clinical relevance of the effects. Finally, consistent with the principles of hypothesis-based WoE, we consider whether the observations from all realms of evidence better support exposure to ambient levels of ozone as a causal factor for adverse effects on atherosclerosis-related biomarkers, or support an alternative explanation (i.e., they do not support causality).

For each aspect of the evaluation, we consider study quality and relevance. Although the studies in each realm have many strengths, they also have methodological limitations, and both can affect the interpretation of their results. Because they have more strengths than limitations, we considered the Tier I studies to be of higher quality and reliability for supporting decisions regarding causation than the Tier II studies. Thus, although we considered the results of all studies, we assigned more weight to the results of Tier I studies in our evaluation. Regarding study relevance, we considered whether study

results are relevant to human exposures to ambient ozone concentrations.

Strength of association

When reported risks are large and precise, there is increased confidence that an association is causal rather than attributable to chance, bias, confounding, or other factors. In general, risk estimates indicating a less than 2-fold change are considered to be weak (Taubes 1995). In the few epidemiology studies of atherosclerosis-related biomarkers that reported risk estimates rather than percent changes in biomarker levels, the sizes of the effects (both positive and negative) were well below 2-fold, and none were statistically significant. For example, in the Tier I study by Lee et al. (2011), the risk estimates for the likelihood of having a CRP level ≥ 8 ng/mL (i.e., a high-risk level for CVD) with increasing ozone exposure across different lag times ranged from 1.05 to 1.49. Similarly, the Tier II study by Poursafa et al. (2011) reported ORs ranging from 1.05 to 1.3 for having "elevated" levels of TF (i.e., in the highest quartile of TF concentrations among study participants) with increasing quartiles of ozone exposure, and from 0.72 to 0.91 for having elevated levels of thrombomodulin.

Although risk estimates of a small magnitude could have a large impact on biomarker levels at the population level because of the widespread exposure to ozone in ambient air, such impacts depend on the existence of a causal relationship between ozone exposure and these outcomes. Because the risk estimates reported in the epidemiology studies of atherosclerosis-related biomarkers are very small in magnitude, they have a higher likelihood of being attributable to other factors besides ozone. Thus, the overall findings do not support a causal relationship between ozone exposure and adverse changes in biomarker levels, and efforts to quantify the impacts of ozone on these outcomes on a population level are questionable and would require explicit acknowledgment of the uncertainty in the causal relationship (Petito Boyce et al. 2015).

Consistency and coherence

Although there are differences in species, exposure parameters, and methods of exposure measurement among the studies we reviewed in each realm of evidence, it is expected that if ozone is a causal factor for adverse changes in atherosclerosis-related biomarkers, the changes should be relatively consistent in the direction of an adverse effect across studies and across categories of biomarkers. Even if some studies did not have sufficient power for the results to reach statistical significance, non-statistically significant changes should be in the same direction across multiple studies, if ozone is a causal factor. In the majority of studies we reviewed, ozone had no statistically significant effect on the biomarkers examined, and there was often no consistency in the direction of the reported effects (both significant and non-significant) for the same biomarkers or those in the same category among studies. An exception to this can be seen with some of the biomarkers of inflammation. Three experimental animal studies by the same group of investigators reported statistically significant increases in TNF-α and decreases in IL-10, and these effects are both indicative of increased systemic inflammation. These changes were measured in normal or ischemic rat heart tissue after exposure to 800 ppb ozone for 28 days, so the relevance to humans is unclear. Although IL-10 was not examined in any human studies, levels of TNF-α were unchanged after ozone exposure in two Tier I controlled human exposure studies and non-significantly *increased* in one Tier II controlled human exposure study; thus, the results for TNF-α in human studies do not corroborate those from experimental animal studies. Levels of CRP, another proinflammatory marker, were non-significantly increased in two (one Tier I and one Tier II) controlled human exposure and two Tier I experimental animal studies, but were either increased or decreased among four Tier I and one Tier II epidemiology studies, indicating that increases in this biomarker may only be associated with the higher ozone exposures used in the former study types. Other biomarkers were not consistently changed in the direction of an adverse effect on inflammation in studies within and among realms (Table 5); therefore, they do not support a causal relationship between ozone exposure and adverse changes in biomarkers of inflammation.

Another exception is biomarkers of oxidative stress. Most of the biomarkers in this category were consistently changed in the direction indicative of an increase in oxidative stress in Tier I and Tier II controlled human exposure studies and experimental animal studies. There were several consistent changes in the same biomarkers across these realms, such as increases in salicylate hydroxylation in Tier I studies, increases in 8-iso-PGF in Tier I and Tier II studies, and increases in 8-OHdG adducts in Tier II studies (Table 6). Most of these changes were also statistically significant. Levels of MDA were also relatively consistently increased among the experimental animal studies and also in the Tier II controlled human exposure study that measured this biomarker. The exposure concentrations at which all of these effects were observed were quite high in the experimental animal studies (≥ 500 ppb ozone), but were much lower in the controlled human exposure studies reporting effects on salicylate hydroxylation (120 ppb) and 8-OHdG adducts (80–103 ppb). One Tier I epidemiology study also examined 8-iso-PGF and reported an increase in this biomarker with ozone exposure, but the three Tier I epidemiology studies that examined 8-OHdG levels reported mixed results, with both significant and non-significant increases and decreases in this biomarker associated with ozone exposure. Together, the Tier I and Tier II controlled human exposure studies and experimental animal studies reported changes in biomarkers indicative of increased oxidative stress with exposure to ozone, mainly at concentrations much higher than ambient levels, and these effects were not fully supported by Tier I epidemiology studies with lower ozone exposures.

There is little evidence for adverse changes in biomarkers of coagulation/vasoreactivity or lipid/glucose metabolism within or across realms of evidence, as studies of these biomarkers did not report consistent or coherent results, even when similar exposure conditions or outcome measurement time points were used. For a large number of these biomarkers, the reported changes were often in the opposite direction of an adverse effect across studies and realms (Tables 7 and 8). There were also no individual biomarkers consistently changed in an adverse direction, across studies and within or among

realms. A possible exception to this is total cholesterol, which was mainly increased in Tier I and Tier II experimental animal studies (with one Tier II study reporting an increase after a 24-h exposure to high ozone concentrations and a decrease after a second exposure period 72 h after the first exposure period ended) and also in humans immediately after controlled exposure to 300 ppb ozone, but not 18 h later.

Biological gradient

Overall, the evidence does not support exposure–response relationships for effects of short-term ozone exposure on atherosclerosis-related biomarker levels. There is no evidence for exposure–response relationships for biomarker changes in epidemiology studies, as the majority of studies reported null results or both increases and decreases in the same biomarker, depending on the lag time. In contrast, the Tier II study by Poursafa et al. (2011) reported an increased trend in TF levels and a decreased trend in thrombomodulin levels with increased ozone exposure.

None of the controlled human exposure studies examined more than one exposure level, with the exception of the Tier I study by Liu et al. (1997), which reported no exposure–response relationship for levels of 2,3-DHBA in participants exposed to 120 or 400 ppb ozone. Liu et al. (1997) did report that the ratio of 2,3-DHBA to 2,5-DHBA was non-significantly increased with exposure to 120 ppb ozone, and significantly increased with exposure to 400 ppb ozone. Because this biomarker, as well as the other biomarkers of oxidative stress, were consistently changed in an adverse direction across the controlled human exposure studies, but not as consistently with the lower-exposure epidemiology studies, this may indicate that there is an exposure–response relationship for this biomarker category. For the few biomarkers that were assessed in more than one controlled human exposure study, effects on the same biomarker across studies did not show an exposure–response relationship.

Several experimental animal studies examined more than one exposure level. There were no exposure–response relationships for the increases in levels of inflammatory biomarkers in the Tier I study by Kodavanti et al. (2011) or the Tier II study by Nachtman et al. (1988). Although Kodavanti et al. (2011) reported slightly increased white blood cell and lymphocyte counts in rats exposed to 1,000 ppb ozone, these biomarkers were slightly lower than control levels at the lower exposure level of 500 ppb ozone. Similarly, neither the Tier I study by Kadiiska et al. (2013) nor the Tier II study by Takatori (1975) reported exposure–response relationships for changes in biomarkers of oxidative stress and lipid metabolism, respectively. Total cholesterol levels were increased at the lower exposure level and decreased at the higher exposure level in the latter study. The Tier I study by Liu et al. (1996) reported an increase in 2,3-DHBA levels with an exposure–response relationship in 2- and 24-month-old rats but not in 9-month-old rats exposed to 1,000 and 2,000 ppb ozone. The Tier I study by Mole et al. (1985) exposed rats to 1,000, 1,750, or 3,000 ppb ozone and reported no exposure–response relationship for increases in triglycerides, and a positive exposure–response relationship for increases in levels of total cholesterol and HDL cholesterol; however, an increase in HDL cholesterol levels is not an indicator of adverse effect on blood lipids and does not increase CVD risk. As with the controlled human exposure studies, there were no exposure–response relationships for the same biomarker across the experimental animal studies. In addition, for the proinflammatory biomarkers (CRP, neutrophil counts, and white blood cell counts), there were relatively consistent increases among experimental animal and controlled human exposure studies, but not in the lower-exposure epidemiology studies of the same biomarker, which may indicate an exposure–response relationship for effects on these biomarkers. Other biomarkers in this category were not consistently changed in an adverse direction in higher-dose studies.

Temporality

Ozone exposure occurs before biomarkers are measured in controlled human exposure and experimental animal studies, but determining exposures in the relevant time frame can be challenging in epidemiology studies. Cross-sectional studies cannot address prior exposures to ozone; while the longitudinal studies of ozone and biomarkers can, they reported results that were inconsistent in direction for all categories of biomarkers, both within and among studies. In addition, the effects reported across studies did not always occur in a consistent time frame, indicating that the results for those biomarkers may be questionable and do not provide strong support for a causal relationship. This was particularly true for the studies of biomarkers of inflammation. For example, CRP levels were significantly increased at lags of 0–1 and 0–2 days in one Tier I study (Chuang et al. 2007), and at a lag of 0 days in another (Bind et al. 2012), but were non-significantly increased or decreased at similar lag times in other Tier I and Tier II studies. White blood cell counts were non-significantly decreased at a lag of 0 and a lag of 1 day in Tier I and Tier II studies, and non-significantly increased at longer lag times (of up to 7 days) in single-pollutant models, but were significantly increased at a lag of 5 days in bi-pollutant models with NO_2 and SO_2. Similar inconsistencies in the time period of effects were also reported across studies of IL-6, the oxidative stress biomarker 8-OHdG, and the coagulation biomarker fibrinogen. Because multiple lag times were examined in these studies but statistical analyses were not adjusted for multiple comparisons, there is an increased likelihood that reported results are attributable to chance.

Specificity

None of the biomarkers examined in the studies we evaluated are specific to ozone or to CVD. Many factors can influence the measured concentrations of certain biomarkers, including time of day, dietary intake patterns, body weight changes, level of physical activity, stress, trauma, or the presence of infection or disease/pre-disease states (Gilstrap and Wang 2012, Zhou et al. 2010, Donde et al. 2012, Navarro et al. 2012). While this may not be an issue under the well-controlled conditions of experimental animal studies, many of these factors were not adjusted for in the analyses in the controlled human exposure and epidemiology studies. Although most of these studies were adjusted for BMI or pre-existing disease, they were

not adjusted for any recent changes in body weight or for the presence of infections. Thus, some of these factors may have been important confounding factors that were unaccounted for, limiting the strength of the evidence for causality from the human studies.

Natural experiments

Only one of the studies in our evaluation qualifies as a quasi-natural experiment. The Tier I epidemiology study by Rich et al. (2012) examined levels of inflammatory and coagulation biomarkers in healthy adults before, during, and after the 2008 Olympic Games in Beijing. Ozone concentrations increased 24% during the Games when air pollution emissions in the city were greatly restricted, and, during this period, circulating concentrations of vWF were significantly decreased, a change that is in the opposite direction of an adverse effect on coagulation. Rich et al. (2012) noted that this seemingly beneficial effect was likely attributable to the negative correlation of ozone with concentrations of NO_2 and other pollutants during the Games. This study does not indicate that ozone adversely impacts coagulation.

Biological plausibility

As noted in the Introduction, the MoA by which short-term exposure to ozone could cause CVD is unknown, but several MoAs with potential biological plausibility have been proposed. Our evaluation of the available data on biomarkers in the proposed pathways of ozone-induced atherosclerosis indicates that although there are consistent and coherent changes in biomarkers of oxidative stress, they do not occur in humans at ambient concentrations. In addition, the reported changes in biomarkers of inflammation, coagulation/vasoreactivity, and lipid/glucose metabolism are less consistent and also not observed at ambient concentrations. The clinical relevance of the mostly small changes in biomarker levels in all categories is unclear. Further, given that the majority of participants in the reviewed epidemiology studies and all participants in the controlled human exposure studies were young, healthy adults, it is unclear how transient effects on biomarker levels in these individuals are relevant to the disease process of atherosclerosis, which occurs over decades and becomes clinically manifest much later in life. Thus, if there is a biologically plausible MoA for ozone-induced CVD, the biomarker data indicate that it is not likely to be *via* the acceleration or exacerbation of atherosclerosis, although additional mechanistic data are needed to confirm this.

Confounding and bias

Confounding and bias are important sources of uncertainty in epidemiology studies. Many co-pollutants, such as PM, have been shown to confound associations between ozone and adverse CV outcomes (e.g., Franklin and Schwartz 2008, Katsouyanni et al. 2009). In addition, as noted above, confounders such as certain lifestyle factors and medical history can affect levels of atherosclerosis-related biomarkers and are conceivably correlated with ozone exposure. In our evaluation of study quality, we scored studies that accounted for these confounders higher than studies that did not. While some studies adjusted for many potential confounding factors, residual confounding (as well as confounding from other factors not considered in the analyses) may contribute to uncertainty in the findings. The majority of Tier I and Tier II studies used single-pollutant models, so confounding by co-pollutants was not addressed. For those studies that used bi- or multi-pollutant models, we found that statistically significant effects were often reduced, no longer statistically significant, or reversed in direction when confounding pollutants were accounted for. This increases the likelihood that the reported effects are attributable to confounding pollutants rather than to a causal relationship with ozone. This issue was particularly apparent in the studies of biomarkers of coagulation, for which the evidence across studies in all realms is weak, given the large number of reported changes that are in the opposite direction of an adverse effect on coagulation.

Selection bias, exposure measurement error, and outcome misclassification are the three main sources of bias in ozone epidemiology studies. Selection bias and outcome misclassification were better controlled for in some studies compared to others. For those studies that we judged to have a higher likelihood of selection bias or outcome misclassification, the direction and magnitude of the potential bias was difficult to discern. Exposure misclassification was possible in all studies, as none used personal exposure measurements, which have a lower potential for exposure measurement error. However, studies that used air monitoring stations within 10 km of participants' residences were likely less biased than those that used area-level monitors. The direction and magnitude of this potential bias was also difficult to discern, as it likely differed across studies, given that personal–ambient ozone correlations can differ based on factors specific to the individual, location, and season. Considering the inconsistency in the direction and magnitude of the changes in biomarker levels across the Tier I and Tier II epidemiology studies for each biomarker category, it is likely that observed effects were at least partially attributable to bias rather than to a causal relationship with ozone.

It is unlikely that confounding and bias had a major impact on the controlled human exposure and experimental animal studies in this evaluation, with the exception of the factors that can influence biomarker levels that were not accounted for in the controlled human exposure studies, as discussed above. Another exception is publication bias, which may be a source of bias in both human and experimental animal studies. Studies with statistically significant results are more likely to be published than those with null findings, leading to published literature that may be unrepresentative of the actual research data generated by investigators (Easterbrook et al. 1991, Siddiqi 2011). Thus, the potential presence of publication bias in the studies of ozone exposure and human health effects may have biased the reported results away from the null.

Clinical relevance

The clinical relevance of the reported effects on biomarker levels among both the Tier I and Tier II studies in each realm is difficult to discern. The changes were quite small in most cases (usually no more than a 10% change), and may be indicative of intra-individual variation or homeostatic (i.e., non-adverse) biological processes rather than atherosclerosis development. Intra-individual variation in levels of biomarkers can make

associations with CVD risk difficult to interpret, particularly when the changes are much smaller than those observed in patients with acute CVD (Gilstrap and Wang 2012).

We compared the range of biomarker levels among study participants in the epidemiology and controlled human exposure studies reporting statistically significant changes to normal reference ranges, and we found that none of the biomarkers in any category with significant and potentially adverse changes exceeded the reference ranges, with the exception of HbA1c. This biomarker was slightly (0.05–0.07%) but significantly increased at all lag times examined in a Tier I epidemiology study (Chuang et al. 2010), and the range of levels in participants (3.5–14.7%) exceeded the reference range of $< 8\%$. The authors did not report the disease status of participants, however, so it is possible that those with HbA1c levels exceeding the range were pre-diabetic or diabetic (conditions for which HbA1c levels are elevated), or that the very small increase in HbA1c levels associated with increasing ozone exposure did not increase their risk of CVD. Indeed, the magnitude of this change is much lower than the changes reported for other biomarkers in each category.

The non-significant changes in biomarker levels in Tier I and Tier II studies across realms were also small in magnitude. Often, both the statistically significant and non-significant changes were in the opposite direction of an adverse effect on atherosclerosis, and were of a magnitude similar to the findings that are consistent with adverse effects. Because it is unlikely that ozone is protective in some studies and harmful in others, even the stronger positive associations may not be indicative of a causal relationship.

Overall, the magnitude of the changes in biomarker levels across studies was generally small, even in the controlled human exposure studies with direct exposure to high concentrations of ozone, and the biomarker levels in study participants did not exceed normal reference ranges in all but one case. This indicates that the changes are likely homeostatic rather than clinically relevant, and do not support a causal relationship between ozone and adverse effects on levels of atherosclerosis-related biomarkers.

Evaluation of alternative explanations

Our integration of the data across realms of evidence indicates that there are many factors that do not support a causal relationship between ambient ozone exposure and changes in levels of atherosclerosis-related biomarkers. Consistent with the principles of hypothesis-based WoE (Rhomberg et al. 2010), we considered two possible explanations for the observations from the biomarker studies and evaluated which explanation is more likely.

The first explanation is that exposure to ambient levels of ozone causes effects on biomarkers that are indicative of increased risk of atherosclerosis and CVD. This explanation is supported by the reported changes in levels of certain atherosclerosis-related biomarkers associated with ozone exposure that are statistically significant and/or consistent in direction across more than one study. It is not supported by results indicating changes in the opposite direction for the same biomarkers or biomarkers in the same category in other studies that we considered to be of similar or higher quality, or the small magnitude and/or unclear clinical relevance of the changes. It is also not supported by the lack of coherence between the human and experimental animal evidence, with the possible exception of effects on biomarkers of oxidative stress and a few biomarkers of inflammation (TNF-α, IL-10, CRP), although this mainly occurs at exposure concentrations much higher than ambient levels. Moreover, it is not supported by the lack of consistently observed exposure–response relationships among studies in each realm. Finally, it is not supported by the unclear relevance of transient changes in biomarker levels to a disease process that takes decades to manifest. To accept this explanation as true, one must accept that short-term exposure to ambient levels of ozone induces adverse changes in levels of atherosclerosis-related biomarkers that are relevant to disease development over decades after exposure, even though this is not supported by the available evidence.

An alternative explanation is that ambient ozone is not a causal factor for effects on atherosclerosis-related biomarkers, and the few positive associations observed in some of the studies are attributable to other factors. This explanation is supported by the lack of relevance of the changes reported at high ozone exposures in controlled human exposure and experimental animal studies to humans exposed to ambient ozone concentrations. It is also supported by the lack of clear clinical relevance of the biomarker changes reported at both higher and lower ozone concentrations in epidemiology studies. This explanation is further supported by the totality of the data across realms of evidence, which provides plausibility for the few changes in the direction of an adverse effect observed in some studies to be deemed false positive results that are likely attributable to chance, bias, or confounding, given the inconsistency in the direction of changes in specific biomarkers as well as in categories of biomarkers across studies. To accept this explanation as true, one must accept that a causal relationship between short-term exposure to ambient ozone levels and adverse effects on atherosclerosis-related biomarkers is not likely in humans.

When assessing the WoE in support of these competing explanations, the first explanation is not adequately supported by the totality of the currently available evidence, and there is more substantial support for the alternative explanation of a lack of a causal relationship.

Causal determination conclusions

We applied the WoE conclusions from Phase 3 to categorize the potential causal relationship between short-term ozone exposure at ambient concentrations and adverse changes in levels of atherosclerosis-related biomarkers. We relied on the four-level categorization of the strength of the overall evidence for or against a causal relationship, proposed by IOM (2008):

Sufficient: The evidence is sufficient to conclude that a causal relationship exists.

Equipoise and Above: The evidence is sufficient to conclude that a causal relationship is at least as likely as not, but not sufficient to conclude that a causal relationship exists.

Below Equipoise: The evidence is not sufficient to conclude that a causal relationship is at least as likely as not, or is not sufficient to make a scientifically formed judgment.

Against: The evidence suggests the lack of a causal relationship.

Our WoE analysis indicates that the evidence does not support a causal relationship because ozone did not alter most biomarker levels in a consistent direction across studies, and the few, consistent changes in the direction of an adverse effect may not be clinically relevant. The changes in biomarkers of oxidative stress and inflammation with coherence across study realms were observed mainly with high ozone concentrations in controlled human exposure and experimental animal studies, and thus, are not relevant to ambient exposures. Moreover, any reported short-term effects on biomarker levels are of uncertain relevance to CVD, a disease that develops over decades.

The studies we reviewed had many strengths, and, in our evaluation of study quality, we judged the majority of studies to be categorized as Tier I studies. Although Tier I studies are of relatively higher quality than Tier II studies, methodological limitations in the Tier I studies are still present. Because of these limitations, the overall database for the potential effects of short-term ozone exposure on changes in levels of atherosclerosis-related biomarkers does not provide definitive evidence regarding a lack of causation. For example, the epidemiology studies are limited in that chance, bias, and confounding cannot be ruled out with confidence. Exposure measurement error may have impacted the findings across the epidemiology studies, as all studies used central-site monitors as surrogates for personal ozone exposure, and ozone exposure can vary substantially in time and space. In addition, while some studies adjusted for many potential confounding factors, residual confounding was possible and may have contributed to uncertainty regarding the interpretation of results. The controlled human exposure studies were conducted mainly at high exposures that do not inform potential causality at current ambient ozone levels. The experimental animal studies are also of limited relevance to humans, given the high ozone exposure concentrations used. The few consistent findings for certain biomarkers among studies within and across realms are of uncertain relevance, given that many factors other than ozone contribute to changes in these markers and that the changes are unlikely to be clinically relevant.

Our evaluation indicates that the totality of the evidence does not support a causal relationship, but when considering study limitations, we conclude that the currently available evidence as a whole is not sufficient to make a scientifically formed judgment regarding a lack of a causal relationship or to conclude that a causal relationship is at least as likely as not. Thus, we categorize the strength of evidence for a causal relationship between short-term exposure to ambient ozone and adverse changes in levels of atherosclerosis-related biomarkers as "below equipoise."

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Supplementary material available online

Supplementary Tables 1–12