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The impact of volatile organic compound exposure on subclinical biomarkers of cardiovascular injury.

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THE IMPACT OF VOLATILE ORGANIC COMPOUND EXPOSURE ON SUBCLINICAL BIOMARKERS OF CARDIOVASCULAR INJURY

By

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B.A., University of Louisville, 2017

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ABSTRACT

THE IMPACT OF VOLATILE ORGANIC COMPOUND EXPOSURE
ON SUBCLINICAL BIOMARKERS OF CARDIOVASCULAR INJURY

Breandon S. Taylor

August 30, 2022

Pollution has been identified as the leading environmental cause of non-communicable disease and premature deaths globally. Volatile organic compounds (VOCs) are gaseous chemical constituents of pollution derived from a variety of sources, including industrial solvents and byproducts, automobile exhaust, tobacco smoke, cleaning supplies, and personal care products. VOCs are also abundant at various Superfund and Hazardous Waste Sites. Emerging data suggest that VOC exposure is associated with several adverse health outcomes, including cardiovascular disease (CVD). VOCs and their metabolites can potentially damage the endothelial lining of blood vessels, resulting in perturbed vascular function and vascular inflammation. We hypothesize that VOC exposure augments CVD risk, which is reflected by significant changes in subclinical biomarkers. To examine the effects of VOC exposures on vascular inflammation, we measured associations between urinary metabolites of VOCs and biomarkers of CVD, including microparticles and circulating angiogenic cells.

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INTRODUCTION

POLLUTION

Pollution is an increasingly growing burden on the Earth and its inhabitants. Both natural and anthropogenic sources of pollution are responsible for the contamination of air, water, and soil. The consequences of pollution are detrimental to economies, the environment, and human health. The Lancet's commission on pollution and health has identified pollution as the leading environmental cause of disease and premature death worldwide (2). The Global Burden of Disease 2015 Study (GBD 2015) estimated that diseases caused by pollution accounted for nearly 9 million premature deaths, or 16% of all deaths around the world (3). More specifically, air pollution is a major contributor of cardiovascular morbidity and mortality. Data from the Institute for Health Metrics and Evaluation (IHME) and World Health Organization (WHO) reveal that global estimated deaths due to air pollution are higher than that of water and soil pollution combined (2). The GBD 2015 study estimated that 4.2 million deaths were due to ambient air pollution, and an additional 2.9 million deaths were associated with household air pollution.

Air pollution is a complex mixture of hazardous substances derived from both anthropogenic and natural sources. It is composed of solid and gaseous components such as particulate matter (PM), ozone (O_3), nitrogen oxides (NO_X), sulfur oxides (SO_X), carbon monoxide (CO), and volatile organic compounds (VOCs). In the 1970s, air pollution was primarily regarded as a respiratory health concern, but data published in recent decades has unmasked its implication in several other diseases including cardiovascular disease, diabetes mellitus, and immune system disorders (4). Extensive research has been conducted to investigate the adverse health effects of $PM_{2.5}$, and

other constituents of air pollution, but not much is known about the impact of volatile organic compounds on cardiovascular disease. For instance, inhalation of fine particulate matter <2.5μm ($PM_{2.5}$) is associated with increased risk for type 2 diabetes (5), hypertension (6), stroke (7), myocardial infarction (8), and cardiovascular mortality (9). While the availability of historical monitoring data has allowed connections between $PM_{2.5}$ and cardiovascular disease to be highlighted, additional parameters must be considered to comprehensively understand the adverse health effects of air pollution exposure. Over 95% of pollutant mass in urban settings consists of gaseous or vaporous compounds, such as volatile organic compounds, which may significantly contribute to disease and mortality (10). In addition to the toxicity elicited by PM2.5, VOCs may also significantly contribute to disease and mortality by adhering to these particulates that individuals are exposed to. Since volatile organic compounds are prevalent constituents of air pollution, it is vital to investigate the levels that individuals are exposed to and how exposure contributes to cardiovascular morbidity and mortality.

VOLATILE ORGANIC COMPOUNDS

Volatile organic compounds (VOCs) are carbon-containing chemicals which exhibit high vapor pressure and low water solubility. Their high vapor pressure at room temperature allows them to readily evaporate into the air. High concentrations of VOCs are generated and emitted into the atmosphere from both biogenic and anthropogenic sources. Although biogenic VOCs are much more abundant worldwide, concentrations of anthropogenic VOCs are usually much higher in urban areas (11). Many people are exposed to VOCs every day in both outdoor and indoor settings. Sources of outdoor VOCs include automobile emissions, industrial facilities, landfills, as well as Superfund and hazardous waste sites. Indoor sources of VOCs arise from sources such as air fresheners, cleaning supplies, paints, solvents, and personal care products. The

Environmental Protection Agency (EPA) claims that concentrations of VOCs indoors are consistently higher than outdoors, and in some cases can be up to ten times higher. With the combined exposure to both outdoor and indoor VOCs, people are subjected to a diverse variety of potentially harmful chemicals.

Volatile organic compounds encompass a wide range of chemical forms including alkanes, alkenes, aromatic hydrocarbons, and oxygenated compounds. The diverse composition among these compounds may result in varying toxicity and concentrations in ambient air. Concentration and duration of an individual's exposure are key factors in determining the toxicity of VOCs. Acute high-level exposure can cause irritation of the eyes, nose, and throat, headache, dizziness, nausea, and dyspnea. Epidemiological studies have indicated that short-term ambient VOC exposure is significantly associated with increased risk of heart failure emergency hospitalizations (12), as well as overall cardiovascular disease related hospitalizations (13).

Chronic low-level exposure to VOCs could manifest serious long-term health effects with no immediate apparent symptoms. Since exposure conditions throughout daily life are variable and individual level exposure is not frequently assessed in the general population, distinguishing clear connections between VOC exposure and health outcomes is complex. Epidemiological studies have demonstrated that chronic exposure to volatile organic compounds is significantly associated with respiratory diseases, liver dysfunction, kidney damage, central nervous system damage, and cancer (14-16). Furthermore, recent evidence suggests that individuals exposed to certain VOCs, like benzene and acrolein, are associated with high cardiovascular disease (CVD) risk (17, 18).

A wide range of animal studies support plausibility that VOC exposure can induce cardiovascular injury and exacerbate cardiovascular disease conditions. Animals exposed to VOCs, such as crotonaldehyde, formaldehyde, and acrolein, exhibited

changes in blood pressure regulation (19-21), decreased circulating stem cells (22), and increased platelet activation (23). Exposure to acrolein has also been shown to induce systemic dyslipidemia, lipoprotein modification (24), dilated cardiomyopathy (25), and accelerate atherogenesis (26). Additionally, other VOCs, like benzene and toluene, have been implicated in cardiovascular toxicity. Benzene exposure induces insulin resistance in mice (27), and toluene exposure significantly increased blood pressure of rats (28). Another airborne VOC, 1,3-butadiene, has been shown to exacerbate the development of atherosclerosis in cockerels (29). Although animal models have served as important tools for understanding adverse cardiovascular effects of some VOCs, real-world human exposure to VOCs can be much more complicated and compounded by several different risk factors. Nonetheless, further assessments of human exposures are required to fully elucidate the impact of volatile organic compounds on vascular inflammation and cardiovascular disease.

CARDIOVASCULAR DISEASE

The World Health Organization (WHO) has estimated that nearly 18 million people die each year from cardiovascular diseases, which accounts for approximately 32% of all deaths worldwide (30). In the United States alone, one person dies every 36 seconds from cardiovascular disease (31). Moreover, 1 in every 4 deaths is due to cardiovascular disease. In addition to the high rates of death, the CDC estimates that cardiovascular disease costs the United States about $219 billion each year. Cardiovascular diseases (CVDs) can be described as a family of ailments that impair proper function of the heart and blood vessels. The most common types of CVDs are coronary artery disease, cerebrovascular disease, peripheral arterial disease, and congestive heart failure, some of which may lead to deep vein thrombosis and pulmonary embolism. Atherosclerosis is a prominent underlying cause of CVDs and can

also potentiate other adverse health events such as myocardial infarction, heart failure, and stroke.

Atherosclerosis is a slow, progressive inflammatory condition that is characterized by lesion formation and luminal narrowing of the arteries due to plaque buildup. The innermost layer of the vascular wall, known as the tunica intima, contains a monolayer of endothelial cells on the luminal side that are connected by adherent tight junctions. This interior lining is crucial for regulating blood pressure, vascular function, and mediating the inflammatory response. Traditional CVD risk factors such as hypertension, smoking, hyperglycemia, and hypercholesterolemia have been historically recognized as contributors to endothelial dysfunction (32-34). Endothelial injury and dysfunction can increase vascular permeability, which allows low-density lipoproteins (LDLs) to enter and become trapped in the sub-endothelial intimal space (35). Once in the sub-endothelial space, LDLs undergo oxidation by reactive oxygen species, free radicals, and enzymes to produce oxidized LDL (ox-LDL). As a response to the damage, endothelial cells become activated to express adhesion molecules that allow circulating monocytes to attach, roll, and transmigrate through the endothelial layer and into the intima via diapedesis (36-38). Next, inflammatory signals induce monocyte differentiation to produce phagocytic macrophages that engulf ox-LDL particles through scavenger receptors to clear them from the intima (39-41). Once the macrophages become laden with cholesterol and lipids, they become foam cells that die and release their contents into the intima. The re-release of cholesterol, ox-LDL, and cell debris back into the intimal space further perpetuates the inflammatory and immune responses. As more LDL crosses into the intima and more leukocytes are recruited to the site of injury, the previously described process is repeated and results in fatty streaks. As a compensatory measure to exposed thrombogenic plaque, smooth muscle cells from the underlying tunica media migrate to the surface of the plaque and produce extracellular matrix to

form a fibrous cap (42, 43). When vascular inflammation persists over time, subendothelial accumulation of lipids, calcium deposits, dead cells and other materials within the intima will continue and eventually lead to the development of atheroma and more complicated lesions (44, 45).

Ultimately, plaque progression in the arterial walls decreases the size of the lumen and disrupts laminar blood flow. Decreased blood flow can prevent sufficient delivery of oxygen and nutrients to target tissues, including the heart. If the heart is not provided with the proper amount of oxygen-rich blood, ischemic heart disease will likely emanate. Narrowing of the arteries due to atherosclerotic plaque buildup will also seriously impair other vital organs such as the lungs, kidneys, brain, and even peripheral limbs. In more advanced lesions, unstable plaques can degrade the fibrous cap and increase vulnerability to rupture. Rupturing of the unstable plaque releases its contents and cap into the circulatory system, which can lead to embolism and thrombotic events that generate life-threatening acute complications such as stroke or myocardial infarction.

As previously mentioned, there are many risk factors for developing CVD including high blood pressure, high cholesterol, diabetes, obesity, unhealthy diet, physical inactivity, smoking tobacco products, excessive use of alcohol, and air pollution (2, 31). Individuals may mitigate their risk for developing CVD by maintaining a healthy lifestyle, but one risk factor that may be difficult to avoid is exposure to airborne volatile organic compounds (VOCs). Upon exposure, VOCs enter the body's circulatory system via inhalation or ingestion. Metabolism of these chemicals by cytochrome P450 enzymes in the liver generate highly reactive metabolites that may be filtered and eliminated by the kidneys or shuttled back into circulation (46). The presence of VOCs and their metabolites in the blood stream have the potential to injure the endothelial layer of the intima to illicit vascular inflammation and endothelial dysfunction (47). Furthermore, a

decrease in repair capacity can exacerbate vascular damage and ultimately contribute to the pathogenesis of atherosclerosis (48). We are currently investigating how VOCs contribute to endothelial injury, vascular dysfunction, and ultimately lead to exacerbation of atherosclerotic plaque progression. To assess the cardiovascular health of individuals, we measured sensitive blood biomarkers (circulating angiogenic cells and microparticles) by flow cytometry.

MICROPARTICLES

Microparticles were first identified by Peter Wolf, in 1967, while performing blood coagulation research with platelets (49). Wolf observed that platelet-removed human plasma contained particulate material which could be separated by ultracentrifugation. He called this particulate material “platelet-dust”. Although Wolf demonstrated the removal of this “platelet-dust” from plasma resulted in coagulation deficiency, it was generally regarded as cellular debris artifact. Since Wolf’s initial discovery of these phospholipid rich particles, the roles of different circulating microparticles have become a topic of interest in several fields of study. Platelet MPs are the most abundant and extensively examined type of microparticles in the circulation of human blood (50). In recent years, microparticles derived from other cell types have been identified and characterized such as endothelial cell MPs. Characterization of non-platelet cell derived microparticles has posed a challenge because they are present in much lower quantities compared to platelet MPs.

Microparticles (MPs) are membrane vesicles, in the submicron range of 0.1 to 1.0μm, which are released from cells as a result of cell activation and/or apoptosis (51). Their size is larger than exosomes, yet smaller than apoptotic bodies. Microparticles have been shown to contain proteins, lipids, and nucleic acids, which allow for cell-cell communication. These extracellularly released vesicles are characterized by

phosphatidylserine on their outer leaflet and vary in composition, characterization, and size depending on the cell type from which they are released (51). For instance, endothelial MPs exhibit expression of membrane-associated proteins like intracellular adhesion molecules (ICAM-1) and vascular cell adhesion molecules (VCAM-1), while lung MPs express angiotensin-converting enzyme (ACE). The current consensus is that most cell types are capable of shedding microparticles in response to apoptosis or cell activation, including cells in circulation, endothelial cells of the vessel walls, and even cells of various organ tissues (i.e., lungs) (52, 53).

Microparticles are believed to be mediators in normal physiological processes, like intercellular signaling and coagulation, to maintain homeostasis within the body (50). They are also important regulators of thrombosis (54), vascular homeostasis (52), and inflammation (50), hence their involvement in cardiovascular health. However, mounting evidence indicates that MPs are implicated in an array of clinically unfavorable conditions and may actively play a role in the pathogenesis of many diseases, such as atherosclerosis. Evidence suggests that microparticles are involved in different stages of atherosclerosis development, and elevated levels of circulating microparticles have been observed in patients with atherothrombotic diseases (51). Patients with classic cardiovascular risk factors like diabetes mellitus (55), acute coronary syndromes (56), hypertension (57), and hypertriglyceridemia (58) also exhibit a marked increase in MP levels.

Importantly, increased levels of leukocyte and endothelial-derived microparticles have been associated with carotid remodeling in individuals before atherosclerosis is even detectable (59). The pathophysiology of circulating microparticles includes endothelial dysfunction, vascular inflammation, and thrombosis. Endothelial-derived MPs are involved throughout atherosclerotic progression, from early stages of acute endothelial injury to extracellular matrix remodeling in complex lesions. High levels of

circulating microparticles can activate the endothelium and effectively induce vascular inflammation by upregulating endothelium-leukocyte adhesion and influencing the polarization of immune cells (i.e., macrophages and monocytes) towards pro-inflammatory phenotypes (60). Additionally, microparticles have been shown to directly impair endothelial function, stimulate endothelial reactive oxygen species (ROS) formation, and induce endothelial inflammation (61, 62). Although the role of endothelial microparticles in thrombosis have not been fully elucidated, they have been identified to express inducible adhesion molecules that could enhance pro-thrombotic conditions. As for their involvement in later stages of atherosclerosis, microparticles have been shown to promote vascular calcification and contribute to vascular remodeling through β1 integrin active enzymes (63, 64). Overall, microparticles could be an inducer of inflammation or may be generated as a byproduct of inflammation.

Various stimuli which lead to the release of microparticles such as modified LDL, HDL cholesterol, proinflammatory cytokines, oxidative stress, and sheer stress have been examined *in vitro* for different cell types (51), but not much is known about the impact of volatile organic compounds on microparticle release. We hypothesize that exposure to VOCs contributes to endothelial activation and injury, resulting in increased microparticle shedding. Perpetual shedding of MPs is believed to evoke prothrombotic and proinflammatory responses (65) which lead to increased MP shedding, vascular inflammation, and further exacerbation of endothelial injury. To address the role of VOC exposure in vascular toxicity, our laboratory has measured endothelial progenitor cell MPs, endothelial MPs, activated endothelial MPs, lung MPs, lung endothelial MPs, and activated lung endothelial MPs in the peripheral blood of human participants as a quantitative measure of injury.

CIRCULATING ANGIOGENIC CELLS

Until the late 1990s, new vessel formation was thought to be a result of endothelial cell proliferation and sprouting from the pre-existing vessels (66). This paradigm was shifted in 1997 when *Asahara et al.* isolated a cell population from human peripheral blood with both progenitor and endothelial cell characteristics (67). This group demonstrated that the isolated cell population differentiated into endothelial cells in vitro, and these cells participated in active angiogenesis in ischemic animal models (67). These cells were coined as 'endothelial progenitor cells' (EPCs) because of their role in postnatal vasculogenesis. Before this discovery, it was widely believed that vasculogenesis only occurred in fetal development during tissue differentiation (66). The revelation of circulating endothelial progenitor cells has changed the way scientists understand remodeling and repair of the adult circulatory system. In recent decades, EPCs have become a growing topic of interest in the field of cardiovascular disease.

Endothelial progenitor cells (EPCs), also known as circulating angiogenic cells (CACs), are bone marrow derived cells that play a critical role in repairing the vascular endothelium after injury (68). Circulating angiogenic cells express surface protein markers of both hematopoietic stem cells and endothelial cells. When the vascular endothelium is damaged by toxicants in the bloodstream or other CVD risk factors, CACs are recruited from the bone marrow and mobilized to the site(s) of injury via the circulatory system. It has been proposed that some subpopulations of CACs may directly incorporate into denuded portions of arteries and differentiate into endothelial cells, while other phenotypically distinct CAC subpopulations may participate in vascular regeneration via paracrine signaling (68). The endothelial regenerative roles of CACs are vital because proper vascular repair can prevent further damage, endothelial dysfunction, and subsequent lesion formation at sites of injury. Although function and repair capacity of different CAC populations is yet to be fully understood, future animal

and population-based studies could provide significant insight for both therapeutic and prognostic potential.

Several clinical studies have reported associations between lower levels of circulating angiogenic cells and cardiovascular disease. Individuals with low levels of one circulating angiogenic cell population, phenotypically described $CD34^{+}/AC133^{+}/CD31^{+}/CD45^{dim}$, had significantly higher incidence of diabetes, higher HbA1c levels, and overall higher CVD risk scores (69). Another study suggests that levels of circulating endothelial progenitors, $CD34^{+}/KDR^{+}$, are predictors of adverse cardiovascular events and death from CVD (70). Moreover, a population study revealed that post-myocardial infarction patients with higher levels of bone marrow derived $CD133^{+}/CD34^{+}$ CACs had significantly greater recovery of left ventricular function (71). Alongside these pieces of evidence, levels of circulating angiogenic cells are associated with many CVD risk factors (72) and may serve as useful biomarkers of vascular function and CVD risk (73).

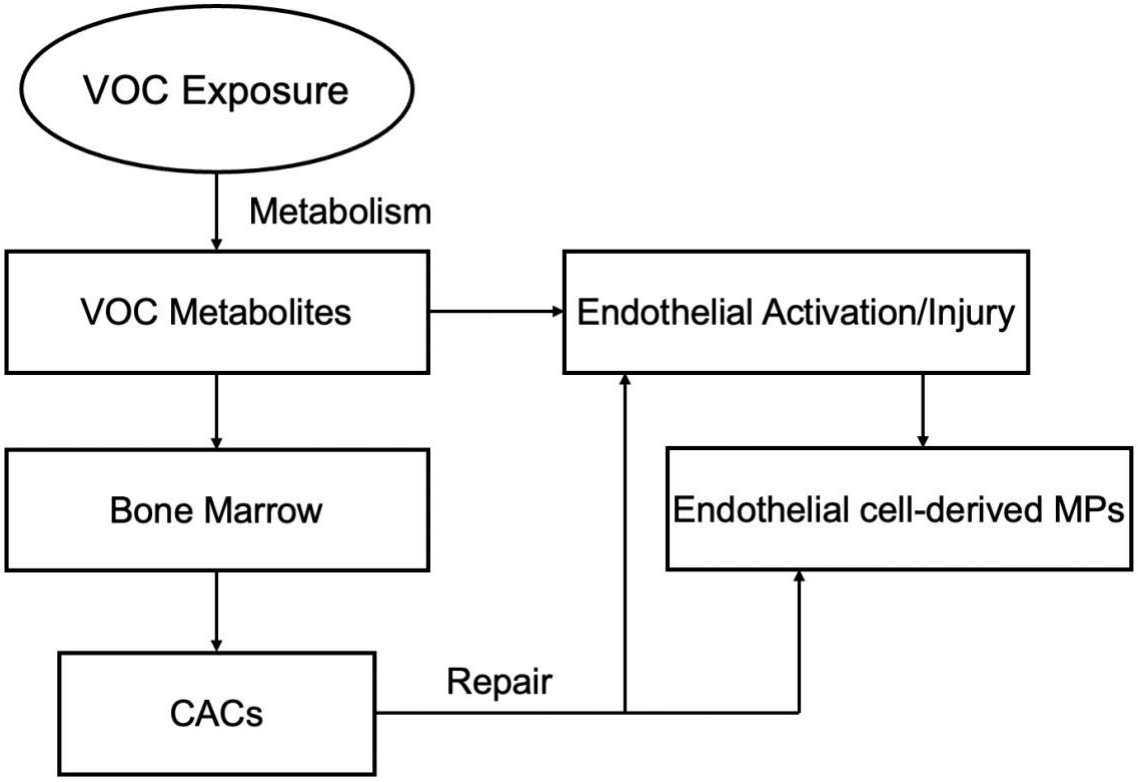
Interpretation of clinical study data for circulating angiogenic cells is complicated due to disease severity and varying levels of CACs at a given time. This can be explained by the biphasic nature of circulating angiogenic cell behavior. The bioactivity of CACs is believed to be modulated by inflammation and oxidative stress (74), which can also influence their recruitment and function. In the event of acute vascular damage, mobilization of CACs from the bone marrow and into circulation is increased to participate in vascular repair. However, the persistence of systemic inflammation is believed to reduce mobilization of CACs from the bone marrow and therefore lower quantities of CACs in circulation. Furthermore, lower levels of CACs in circulation can also be explained by their selective accumulation in chronically inflamed tissue, as observed in patients with rheumatoid arthritis (75). Evidence from clinical studies linking CACs to cardiovascular and inflammatory diseases supports this biphasic response

relationship. Low quantities of CACs in peripheral blood have been observed in patients with coronary artery disease (CAD) (70), rheumatoid arthritis (RA) (76-78), and systemic lupus erythematosus (SLE) (79, 80) compared to healthy control individuals. In the context of heart failure (HF) patients, increased levels of CACs have been observed in early disease stages, while CAC quantities decrease with the severity and progression of HF (81). However, systemic and thorough evaluation of VOCs on biomarkers of vascular inflammation is lacking.

Given their putative role in maintaining vascular health, CACs have been evaluated to examine how airborne toxicant exposure contributes to endothelial dysfunction and CVD pathogenesis. Epidemiological studies have shown that long-term tobacco use depletes levels of CACs compared to non-smoking individuals and cessation from smoking has the potential to restore CAC levels to a certain degree (82, 83). Furthermore, smoking has been shown to significantly impair differentiation and functional activities of CACs, which are vital for vascular homeostasis (84). Aside from tobacco smoke, which contains high levels particulate matter (PM) and several VOCs (i.e. acrolein, benzene, styrene, ethylbenzene, toluene, and xylene) (85), individuals are exposed to a plethora of constituents in ambient air pollution that are detrimental to endothelial function (86). Exposure to fine particulate matter, with an aerodynamic diameter <2.5μm ($PM_{2.5}$), has been identified to suppress circulating levels of CACs, increase platelet activation, and increase levels of high-density lipoprotein in human plasma (87). Though the mechanism by which $PM_{2.5}$ contributes to endothelial injury in humans remains unclear, exposure to $PM_{2.5}$ prevents the mobilization of hematopoietic progenitor cells from bone marrow in mice (88). Acrolein and benzene, two VOCs commonly present in air pollution, have also been shown to decrease levels of circulating hematopoietic progenitor cells in experimental animals (22, 89, 90). Overall, circulating angiogenic cells have been utilized as biomarkers in several studies

assessing endothelial dysfunction and cardiovascular disease. To investigate the effects of VOC exposures on vascular repair, we measured the associations between 16 urinary VOC metabolites and 15 different CAC subtypes in the peripheral blood of non-smokers with low to severe CVD risk. For disclosure, the data for VOCs and CACs has been published and I am a coauthor on the publication (1). We hypothesize that exposure to volatile organic compounds prolongs endothelial injury by depleting circulating angiogenic cells.

Graphical Abstract.



METHODS AND MATERIALS

STUDY POPULATION AND DESIGN

Adult study participants residing in Louisville, KY and in the age range of 25-70 years were recruited for this cross-sectional study. Participants were enrolled during the summer months (May through September) of 2018 and 2019 to reduce variability of VOC heterogeneity due to seasonal changes. Individuals diagnosed with hepatitis, HIV/AIDS, currently undergoing cancer treatment, or other severe comorbidities were excluded from this study. People weighing less than 100 pounds, prisoners, pregnant women, and other vulnerable populations were also excluded. Willing adults who met the enrollment criteria provided informed written consent and agreed to the collection of blood and urine specimens at the clinical site.

A questionnaire was utilized to acquire demographic information, including residential home address, age, sex, ethnicity, smoking status, and body mass index (BMI). Smoking status and tobacco use was confirmed by measuring urinary concentrations of cotinine. The questionnaire completed by study participants also provided information about current treatment and medication usage, blood pressure, CVD risk, history of CVD, and overall health. Hypertension status was defined by previous diagnosis or systolic blood pressure greater than 140mmHg, while diabetes status was defined by previous diagnosis or HbA1c levels greater than 6.5%. Privacy and security of all participant information was maintained with the discretion of HIPAA and in compliance with federal law. This study was approved by the University of Louisville Institutional Review Board (IRB 15.1260).

In total, 735 eligible participants met inclusion criteria and were enrolled in the study. Out of these 735 participants, some were unable to provide peripheral blood samples, urine samples, or had missing relevant covariates. The total number of participants we were able to include was 603 for the CAC analysis (1), and 667 for the MP analysis. To control for tobacco-derived VOCs in the non-smoker analyses, participants with urinary cotinine levels >40µg per gram creatinine were considered smokers/tobacco users.

URINARY METABOLITES OF VOLATILE ORGANIC COMPOUNDS

Study participants provided a non-fasting spot urine sample at the time of their visit. Upon collection, samples were immediately transferred to 4°C and transported to the University of Louisville for long-term storage (-80°C) and analysis. To assess VOC exposure at the individual level, 16 metabolites of 12 VOCs were quantified by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) using methods described by Lorkiewicz et al (91, 92). Nicotine metabolite, cotinine, was also quantified by UPLC-MS/MS to verify smoking status of participants. Analysis of urinary VOC metabolites was performed using the ACQUITY UPLC core system and a Quattro Premier XE triple quadrupole mass spectrometer coupled with an electrospray source (Waters, Inc). Urinary creatinine levels were measured using a COBAS MIRA-plus analyzer (Roche) with Infinity Creatinine Reagent (Thermo Fisher Scientific). Values obtained for urinary analytes were normalized to the individuals' urinary creatinine levels. VOC metabolites with concentrations below the limit of detection (LOD) were divided by the square root of 2 for statistical analysis purposes.

FLOW CYTOMETRY

Microparticles and circulating angiogenic cells were measured by flow cytometry. Fresh peripheral blood samples were obtained from participants at the time of their study visit. Blood was collected in a conventional glass BD Vacutainer® Mononuclear Cell Preparation Tube (CPT) (Becton Dickinson; NJ) containing 0.1M sodium citrate anticoagulant. Upon same-day arrival to the laboratory, the samples were centrifuged at 1600 x g for 30 minutes at room temperature and stored upright at room temperature overnight. The following morning, plasma from the upper layer was decanted into a 15mL conical tube containing 7mL phosphate-buffered saline (PBS) and centrifuged at 500 x g for 5 minutes at room temperature. After centrifugation, a 1mL aliquot of specimen supernatant was placed in a 1.5mL Eppendorf tube and stored on ice for microparticle staining. The microparticle staining protocol is described in a later section following the circulating angiogenic cell staining protocol. The remaining supernatant was discarded, and the cell pellet was used for staining circulating angiogenic cells.

Circulating Angiogenic Cells:

Cell pellets were washed twice with 1% bovine serum albumin (BSA)/PBS buffer and incubated with Human FcR Blocking Reagent (Miltenyi Biotec) for 10 minutes at 4°C to enhance specificity of immunofluorescent staining. After blocking, the cells were stained with a cocktail of fluorescently conjugated antibodies against targets CD14 (monocyte, macrophage), CD34 (endothelial progenitors), CD45 (hematopoietic cells), CD146 (endothelial cells), and AC133 (early hematopoietic endothelial progenitors) for 30 minutes at 4°C. Fluorescent antibodies included FITC-AC133 (Miltenyi), PerCP-CD14 (Miltenyi), VioBlue-CD34 (Miltenyi), VioGreen-CD45 (Miltenyi), and APC-CD146 (Miltenyi). The stained cells were then washed with 1% BSA/PBS, centrifuged at 500 x g for 5 minutes, and resuspended in a fixed volume of 1% BSA/PBS. Events were

collected by fluorescence-activated single cell sorting (FACS) using an LSR II flow cytometer (BD Biosciences) for two minutes at high-speed setting. Unstained controls were used to set positive and negative boundaries for gating. The monocyte/macrophage marker CD14 was used as an exclusion marker, and CAC populations were defined by their differential expression of surface markers CD34, CD45, CD146, and AC133. To correct for variation among samples, CACs were normalized to the total number of $CD14^-$ cells in each sample. Fifteen subtypes of circulating angiogenic cells were isolated, fluorescently labeled, and quantified. Classification of circulating angiogenic cell populations is included in Supplemental Table 1.

Circulating Microparticles:

The 1mL aliquot of PBS-diluted plasma was centrifuged for 2 minutes at 11,000 x g using a fixed angle rotor centrifuge in the cold room. Next, the supernatant was transferred to a new autoclaved 1.5mL Eppendorf tube and centrifuged for 45 minutes at 17,000 x g. After high force centrifugation, the supernatant was aspirated and the pellet was resuspended in 3% FBS/7.5mM Ca^{2+} PBS. The samples were incubated with Human FcR Blocking Reagent for 10 minutes at 4°C before the addition of antibodies. Three tubes were prepared for each microparticle sample. Tube 1 contained only buffer and microparticle suspension to serve as an unstained control. Tube 2 was used to stain for platelet MPs, endothelial progenitor cell (EPC) MPs, endothelial MPs, and activated endothelial MPs using fluorescently conjugated antibodies APC-CD34 (BD Biosciences), PE-Cy7-CD41 (BD Biosciences), and PE-CD144 (Thermofisher). Tube 3 was used to stain lung MPs, lung endothelial MPs, and activated lung endothelial MPs using APC-CD143 (Biolegend), PE-CD144, and PE-Cy5-CD62E (BD Biosciences). Tube 2 and tube 3 were both stained with Annexin V Pacific Blue conjugate (Thermofisher) to indicate

apoptosis via binding externalized phosphatidylserine. Samples containing microparticle suspension and fluorescently labeled antibodies were incubated for 30 minutes at room temperature in the dark. Following the 30-minute incubation period, 1% FBS/2.5mM Ca^{2+} PBS was added to each sample tube to stop the reaction. 10μm polystyrene beads were added to each of the three sample tubes and two separate suspensions were prepared for 1μm beads and 10μm beads. Events were collected using an LSR II flow cytometer on the low-speed setting for 10,000 events. Microparticles were normalized to the count of 10μm beads and 1μm beads were used for gating exclusion. Classification of the cell derived microparticles measured is included in Supplemental Table 2.

AIR POLLUTION AND METEOROLOGICAL DATA

Air pollution data for ambient levels of $PM_{2.5}$ ($\mu g/m^3$) and ozone (ppm) were obtained from three regional EPA-validated monitoring stations in the Louisville, Kentucky region. We used the average of the three monitors for the day study participants provided blood and urine specimens.

Meteorological data on average daily temperature (°F) and relative humidity (%) were obtained from the National Oceanic and Atmospheric Administration's (NOAA) National Centers for Environmental Information. Data was downloaded for the Louisville International Airport monitoring station, which is near the residence of study participants, for the day study participants provided blood and urine samples. This is publicly available data (https://www.ncdc.noaa.gov/cdo-web/datatools/selectlocation).

STATISTICAL ANALYSIS

Participant characteristics are expressed as mean ± standard deviation (SD) for continuous variables, and categorical variables are expressed as frequency (%).

Descriptive statistics were conducted for all VOC metabolites. Although circulating angiogenic cells (CACs) and microparticles (MPs) were analyzed independently, the overall statistical analysis approach was applied to both CACs and MPs. Since CACs were positive and heavily right-skewed, generalized linear models with the gamma distribution and log-link were used to examine associations between CACs and VOC metabolites. The same analytical approach was applied to examine associations between MPs and VOC metabolites. Model adjustments for full data set included: age, sex, race, BMI, diabetes status, hypertension status, daily $PM_{2.5}$ levels, and smoking status.

We examined a total of 240 unique comparisons between CACs and VOC metabolites and 96 unique comparisons between MPs and VOC metabolites. The Benjamini-Hochberg procedure was applied to account for multiple testing and to control for false-discovery rate (FDR). For both CAC and MP analyses, we measured associations for the total study participant group, as well as the subgroup of non-smoking participants.

ANALYSIS OF NON-SMOKERS

Models for non-smokers (urinary cotinine <40 μg/g creatinine) were adjusted for age, race, sex, BMI, diabetes status, hypertension status, and daily $PM_{2.5}$ levels. In non-smokers, there was minimal correlation between VOC metabolites and average daily ambient $PM_{2.5}$, ozone, temperature, and relative humidity. Environmental risk scores (ERSs) representing cumulative VOC exposure risk were calculated for each MP type and each CAC separately utilizing a modified version of the method developed by Park *et al.* (93). Steps for generating ERS are as followed: (1) VOC metabolites were log transformed, (2) each log-transformed VOC metabolite was regressed against each MP and each CAC separately and adjusted for covariates, (3) the environmental risk score

for each participant was calculated as the sum of log-transformed VOC metabolite x (regression coefficient/standard error). If multiple VOC metabolites originate from the same parent compound, only one was used to generate the ERS. The VOC metabolites used to calculate ERSs were 3HPMA, AAMA, CYMA, MU, MHBMA3, HPMMA, AMCC, PGA, 2HPMA, MA, BMA, and 3MHA+4MHA. We tested interactions between covariates and log-transformed VOC metabolites. Analyses were stratified by participant characteristics to determine whether effect estimates differed by subgroups.

Since the nature of circulating angiogenic cells may follow a biphasic response, potential non-linear relationships between CACs and VOC metabolites were examined using restricted cubic splines. Tests for non-linearity used the likelihood ratio test. All statistical analyses were performed using SAS, version 9.4 (SAS Institute Inc.; NC) and GraphPad Prism, version 8 (GraphPad Software; CA).

RESULTS

Associations Between Volatile Organic Compounds and Microparticles:

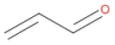
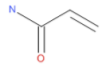
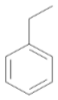
Circulating microparticles (MPs) were positively associated with several urinary metabolites of volatile organic compounds (VOCs). For microparticle analyses, demographic characteristics of study participants are summarized in Table 1, and urinary levels of VOC metabolites are listed in Table 2.

Table 1. Participant Characteristics for Microparticle Analyses

Variable	Total (n = 667)	Non-smokers (n = 413)	Smokers (n=254)	p-value
Sex, Male	268 (40%)	151 (37%)	117 (46%)	0.015
Race				<0.001
White	517 (78%)	343 (83%)	174 (69%)	
Black	115 (17%)	48 (12%)	67 (26%)	
Other	35 (5%)	22 (5%)	13 (5%)	
Age (years)	49.5 ± 12.8	50.4 ± 13.3	48.2 ± 11.8	0.033
BMI (kg/m^2)	30.4 ± 6.9	30.9 ± 7.1	29.6 ± 6.6	0.016
SBP (mmHg)	128.0 ± 17.5	128.3 ± 17.8	127.6 ± 17.2	0.630
DBP (mmHg)	78.7 ± 11.5	78.0 ± 11.1	79.8 ± 12.1	0.051
Hypertension	282 (42%)	178 (43%)	104 (41%)	0.584
Diabetes	130 (19%)	73 (18%)	57 (22%)	0.131
MI	41 (6%)	26 (6%)	15 (6%)	0.903
Medication Use				
ASA	75 (12%)	50 (13%)	25 (11%)	0.421
Statin	94 (15%)	68 (17%)	26 (11%)	0.034
ACE or ARB	99 (16%)	72 (18%)	27 (12%)	0.024
Beta Blockers	57 (9%)	37 (9%)	20 (9%)	0.720
Daily $PM_{2.5}$ (ug/m^3)	8.8 ± 3.4	8.7 ± 3.4	9.1 ± 3.4	0.166
Daily Ozone (ppm)	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.543

Values represent mean ± standard deviation for continuous variables, and frequency (%) for categorical variables. P-values are based on t-test for continuous variables, and Chi-squared test for categorical variables. P-values represent the difference in demographic variables between the two groups: non-smokers and smokers. Abbreviations: BMI indicates body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MI, myocardial infarction; ASA, aspirin; ACE, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; $PM_{2.5}$, particulate matter with diameter <2.5 micrometers.

Table 2. Summary Statistics of urinary VOC metabolites and Parent Compound for Microparticle analyses

Parent Structure	VOC	Metabolite	Total Mean ± SD	Non-Smokers Mean ± SD	n (%) LOD	Smokers Mean ± SD	n (%) LOD
	Acrolein	CEMA	194.7 ± 218.8	114.2 ± 113.4	43 (10.4%)	325.5 ± 278.2	9 (3.5%)
		3HPMA	871.6 ± 1218.1	340.2 ± 344.5	0 (0%)	1735.7 ± 1581.8	0 (0%)
	Acrylamide	AAMA	108.4 ± 119.0	72.0 ± 64.4	1 (0.2%)	167.6 ± 157.7	0 (0%)
	Acrylonitrile	CYMA	59.2 ± 107.3	6.3 ± 21.6	254 (61.5%)	145.2 ± 132.4	8 (3.2%)
	Benzene	MU	149.2 ± 198.4	136.7 ± 219.3	6 (1.5%)	169.6 ± 157.2	4 (1.6%)
	1,3-Butadiene	DHBMA	404.1 ± 211.3	353.2 ± 174.8	0 (0%)	487.0 ± 237.9	0 (0%)
		MHBMA3	28.4 ± 39.4	8.9 ± 8.0	19 (4.6%)	59.9 ± 48.6	1 (0.4%)
	Crotonaldehyde	HPMMA	763.1 ± 945.9	306.2 ± 297.2	1 (0.2%)	1506.1 ± 1147.3	0 (0%)
	N,N-Dimethylformamide	AMCC	296.8 ± 290.0	161.7 ± 118.1	5 (1.2%)	516.5 ± 347.1	0 (0%)
	Ethylbenzene, styrene	PGA	299.8 ± 212.5	224.7 ± 142.6	12 (2.9%)	421.3 ± 248.1	4 (1.6%)
	Propylene oxide	2HPMA	81.2 ± 340.1	56.9 ± 119.2	4 (1.0%)	120.7 ± 528.0	2 (0.8%)
	Styrene, ethylbenzene	PHEMA	2.8 ± 3.9	2.2 ± 3.9	189 (45.8%)	3.7 ± 3.8	42 (16.5%)
		MA	321.6 ± 249.4	232.6 ± 163.7	1 (0.2%)	465.7 ± 293.3	0 (0%)
	Toluene	BMA	11.5 ± 12.5	11.3 ± 11.7	4 (1.0%)	11.7 ± 13.6	0 (0%)
	Xylene	2MHA	56.2 ± 94.0	32.0 ± 72.4	60 (14.5%)	95.3 ± 110.6	7 (2.8%)
		3MHA+4MHA	426.2 ± 667.7	208.5 ± 396.8	30 (7.3%)	777.5 ± 844.5	4 (1.6%)

VOC metabolites were measured in 413 non-smoking participants, 25-70 years of age with low to severe CVD risk. IQR, interquartile range; LOD, limit of detection. Metabolite units: µg/g creatinine. The full chemical name and LOD for each metabolite is listed in *Supplemental Table 1.*

On average, smokers had higher levels of urinary VOC metabolites compared to non-smokers and both groups had a high degree of variability. Several urinary VOC metabolites were positively associated with levels of MPs measured in peripheral blood (Figure 1). Pair-wise associations were calculated for the total group of participants (Figure 1A) and for the stratified group of non-smoking participants (Figure 1B). Microparticles derived from all cell types measured - endothelial progenitor cell (EPC)

MPs, endothelial MPs, activated endothelial MPs, lung MPs, lung endothelial MPs, and activated lung endothelial MPs – were significantly associated with multiple urinary VOC metabolites. Of all the MP subtypes, the most significant associations were observed in EPC MPs and endothelial MPs, which suggests they could be the most sensitive targets of VOC toxicity.

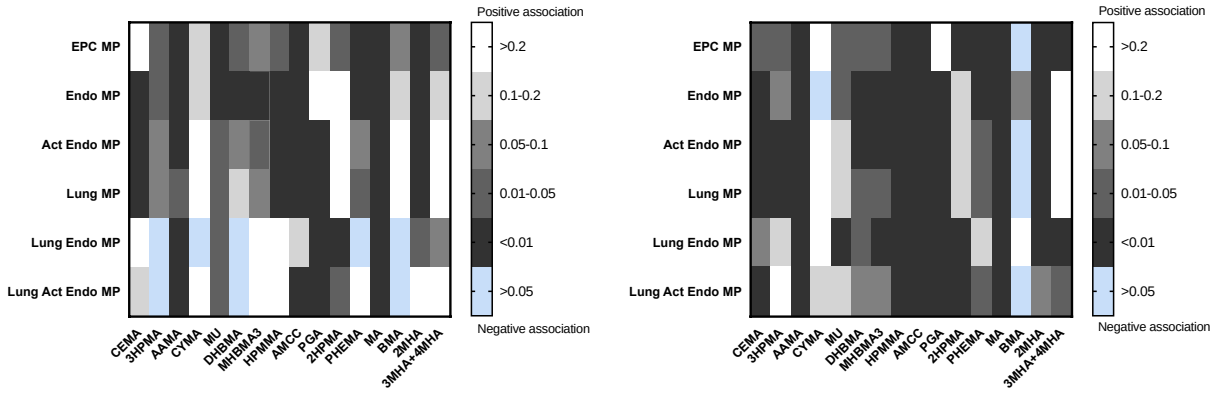


Figure 1. Heat map of pair-wise associations between the urinary metabolites of VOCs and microparticles. (A) False Discovery rate adjusted p-values for total sample (n=667). (B) False Discovery rate adjusted p-values for non-smokers (n=413). Generalized linear models were adjusted for age, sex, race, BMI, diabetes, hypertension, daily ozone, and daily $PM_{2.5}$ levels. Total samples was additionally adjusted for smoking status. Black grids represent positive associations and blue grids represent negative associations. The intensity of colors represent p-values, with darker grids indicating smaller p-values.

Since smokers had much higher levels of exposure to VOCs compared to non-smokers, we focused our subsequent analyses on non-smokers to determine how lower levels of exposure are associated with markers of endothelial damage. We created an Environmental Risk Score (ERS) for each type of microparticle to visualize the extent to which total VOC exposure may influence the abundance of different microparticle types (Figure 2). Strong positive risk scores (>150) were observed for all microparticles measured. This indicates that the levels of microparticles in circulation are positively associated with the urinary metabolites of VOCs overall, regardless of the MP's cellular origin.

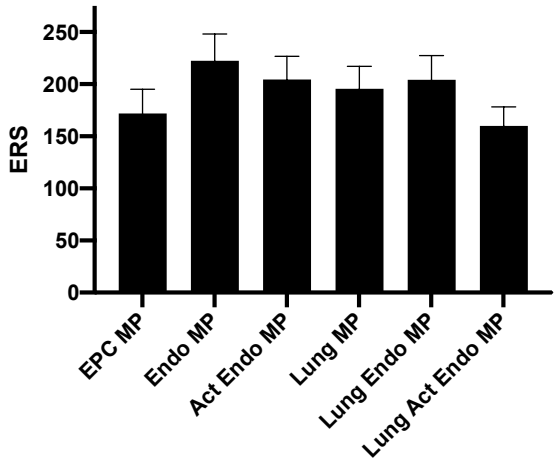


Figure 2. Association between Environmental Risk Scores and Microparticles in non-smokers. Environmental Risk score was calculated for each cell as follows: 1. VOC metabolites were log transformed. 2. Each log transformed VOC metabolite was regressed against microparticles separately, while adjusting for covariates. 3. ERS was calculated as the sum of the log transformed VOC x (regression coefficient/standard error). Mean and standard deviation of the ERS was calculated for each Microparticle. Represents mean and standard deviation of environmental risk score for each cell.

To examine how different microparticles may be influenced by individual VOCs, we measured independent associations between VOC metabolites and MPs while adjusting for other VOC exposures (Figure 3). These generalized linear models were adjusted for age, sex, race, body mass index (BMI), diabetes, hypertension, daily ozone levels, and daily $PM_{2.5}$ levels. The values represent percent difference per 2-fold increase in VOC metabolites. We found that endothelial MPs were positively associated with metabolites of aromatic hydrocarbons benzene (MU), ethylbenzene (PGA), styrene (PHEMA), and xylene (2MHA). Endothelial MPs were also positively associated with the metabolite of acrylamide (AAMA). Microparticles classified as activated endothelial MPs (<1µm, Annexin $V^+/CD144^+/CD62E^+$) showed significant positive associations with crotonaldehyde (HPMMA), N,N-Dimethylformamide (AMCC), ethylbenzene (PGA), and xylene (2MHA). Similar to endothelial MPs and activated endothelial MPs, endothelial progenitor cell (EPC) derived microparticles, or EPC MPs, were positively associated with xylene (2MHA). The consistent positive associations with xylene observed among

endothelial MPs, activated endothelial MPs, and EPC MPs suggests that exposure to xylene is intimately related to endothelial activation and injury.

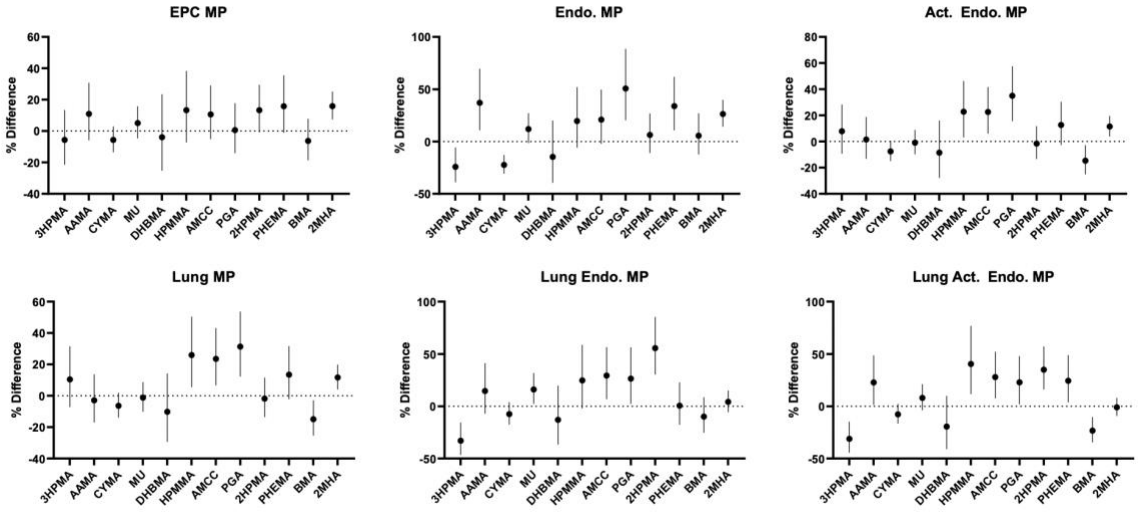


Figure 3. Association between microparticles and multiple VOC metabolites in the same model for non-smokers. Values represent % difference per 2-fold increase in VOC metabolites. Models adjusted for age, sex, race, BMI, diabetes, hypertension, daily ozone levels, and daily PM2.5 levels. Represents independent association between VOC metabolites and MPs while adjusting for other VOC exposures.

We also observed significant associations with lung-derived microparticles in circulation; lung MPs, lung endothelial MPs, and activated lung endothelial MPs. Lung MPs were positively associated with urinary metabolites of crotonaldehyde (HPMMA), N,N-dimethylformamide (AMCC), ethylbenzene (PGA), and Xylene (2MHA). Microparticles originating from lung endothelial cells, or lung endothelial MPs, were positively associated with levels of benzene (MU), N,N-dimethylformamide (AMCC), ethylbenzene (PGA), and propylene oxide (2HPMA) urinary metabolites. Moreover, we found that activated lung endothelial MPs had significant positive associations with the most urinary VOC metabolites. Activated lung endothelial MPs were positively associated with acrylamide (AAMA), crotonaldehyde (HPMMA), N,N-dimethylformamide (AMCC), ethylbenzene (PGA), propylene oxide (2HPMA), and styrene (PHEMA) metabolites. The associations observed for lung endothelial MPs and activated

endothelial MPs were similar, with the exception that activated lung MPs were positively associated with more of the urinary VOC metabolites. This may suggest that activated lung MP are the most sensitive to volatile organic compound exposure. Overall, N,N-dimethylformamide (AMCC) and ethylbenzene (PGA) metabolites were positively associated with all subtypes of lung MPs that were measured. These observations suggest that exposure to ambient levels of volatile organic compounds (VOCs) sufficiently causes pulmonary toxicity in a quantifiable manner.

Subgroup analyses revealed sex-dependent, age-dependent, and BMI-dependent significant interactions between microparticles (MPs) and urinary VOC metabolites. To examine whether the associations were modified by demographic characteristics of the participants, we performed subgroup analyses that were stratified by sex, age, and BMI (Figure 4). As previously described, generalized linear models were adjusted for age, sex, race, BMI, diabetes, hypertension, daily ozone levels, and daily $PM_{2.5}$ levels unless the covariate was used for stratification. Values represent percent difference per 2-fold increase in VOC metabolites. We found several sex-dependent associations among the six microparticle subtypes and sixteen urinary VOC metabolites that we measured. For female participants, we consistently observed higher levels of 1,3-butadiene (DHBMA) urinary metabolite in relation to EPC MPs, endothelial MPs, activated endothelial MPs, and lung MPs compared to males. Additionally, in females, we found significant interactions between the urinary metabolite of acrylonitrile (CYMA) and EPC MPs, endothelial MPs, and activated lung endothelial MPs compared to males. For male participants, we observed the most significant interactions with the urinary metabolite of toluene (BMA), which was positively associated with EPC MPs, lung endothelial MPs, and activated lung endothelial MPs.

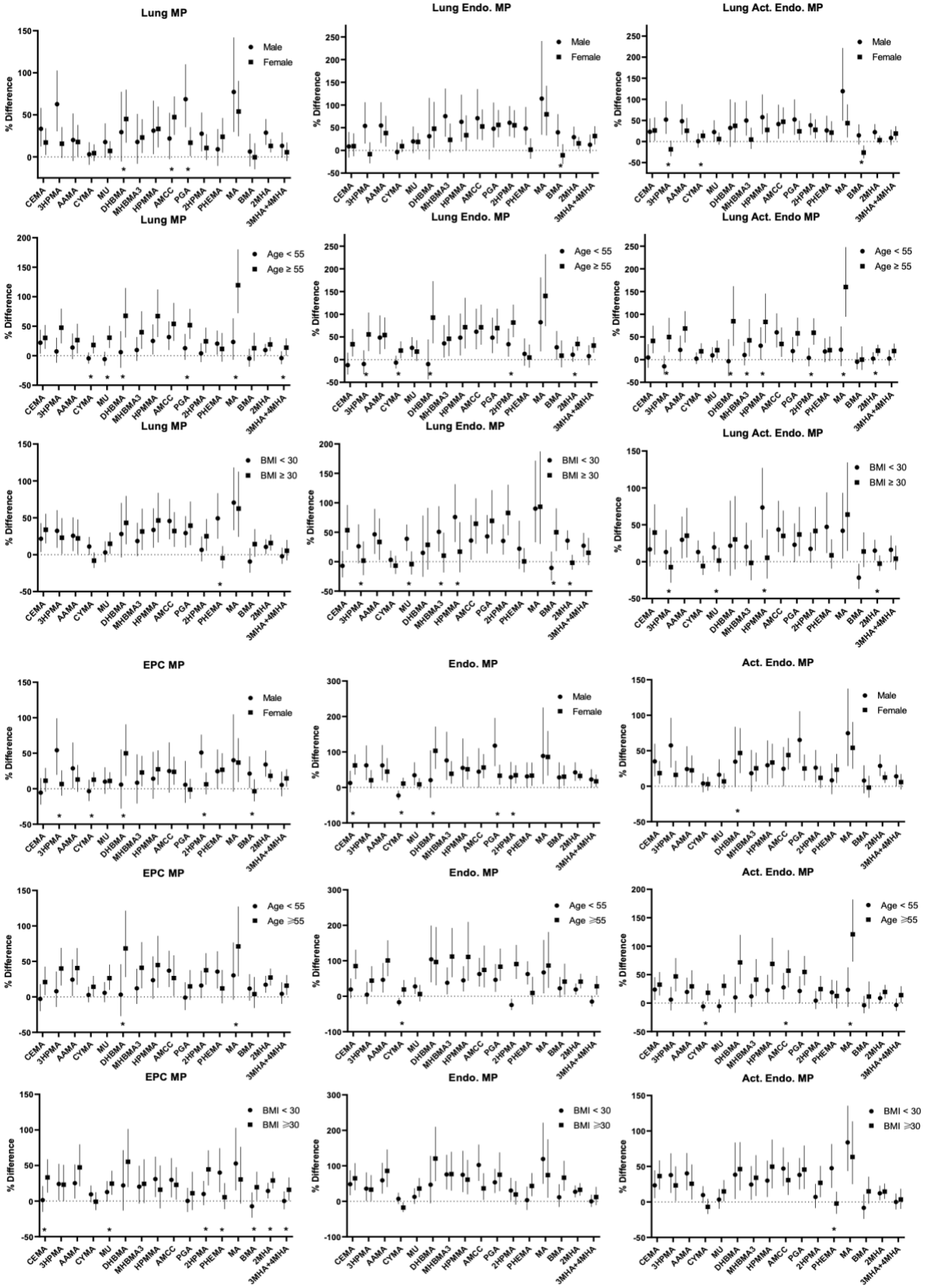

Lung MP
Lung Endo. MP
Lung Act. Endo. MP
Male
Female
Age < 55
Age ≥ 55
BMI < 30
BMI ≥ 30
EPC MP
Endo. MP
Act. Endo. MP
Age ⩾55
BMI ⩾30
% Difference
CEMA
3HPMA
AAMA
CYMA
MU
DHBMA
MHBMA3
HPMMA
AMCC
PGA
2HPMA
PHEMA
MA
BMA
2MHA
3MHA+4MHA

Figure 4. Subgroup analysis of the relationship between different microparticles and VOC metabolites. Generalized linear models adjusted for age, sex, race, BMI, diabetes, hypertension, daily ozone levels, and daily PM2.5 levels, unless the covariates were used for stratification. *Significant interaction between subgroups in the full model.

When the associations between MPs and VOC metabolites were stratified by age, we found that older participants (≥ 55 years) clearly displayed a higher percent difference across the board compared to younger participants (<55 years). This follows the logical presumption that older individuals, in general, have a higher degree of cardiovascular injury than younger individuals. Although we observed a modest number of significant interactions for endothelial-associated MPs (i.e., EPC MPs, endothelial MPs, activated endothelial MPs), the significant interactions for lung-associated MPs were more numerous. Compared to younger participants, older participants had significant interactions for the 1,3-butadiene urinary metabolite (DHBMA) among all lung-derived microparticles (lung MPs, lung endothelial MPs, and activated lung endothelial MPs). The significant interactions of the age comparison associations suggests that older individuals are likely more vulnerable to the cardiovascular toxicity of volatile organic compounds (VOCs), which is reflected by a greater abundance of microparticles (MPs).

Since elevated body mass index (BMI) is a significant risk factor for cardiovascular disease, we also performed a subgroup analysis that stratified participants into two groups (BMI <30 and BMI ≥30). Although we expected that obese individuals (BMI ≥30) would have a greater abundance of MPs in circulation, the results were mixed. On average, participants with BMI ≥30 had higher levels of endothelial progenitor cell microparticles (EPC MPs) while participants with BMI <30 had higher levels of lung endothelial MPs and activated lung endothelial MPs.

Associations Between Volatile Organic Compounds and Circulating Angiogenic Cells:

The levels of circulating angiogenic cells (CACs) in peripheral blood were significantly associated with the levels of several urinary VOC metabolites in single-VOC analyses. For CAC analyses, demographic characteristics of study participants are summarized in Table 3, and levels of urinary VOC metabolites are listed in Table 4. Pair-wise associations were calculated for the total group of participants (Figure 5A) and for the subgroup of non-smoking participants (Figure 5B). In our single-VOC analysis, we found mostly negative associations between CACs and urinary VOC metabolites along with a couple of positive associations. In the full data set containing all participants, PGA- a metabolite derived from ethylbenzene or styrene- was negatively associated with CAC-3, 5, 6, and 15.

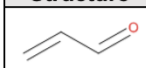
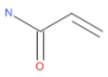
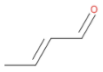
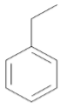
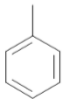
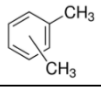
Table 3. Participant Characteristics for Circulating Angiogenic Cell Analyses

Variable	Total (n=603)	Non-Smokers (n=375)	Smokers (n=228)	p-value
Sex, Male	242 (40%)	135 (36%)	107 (47%)	0.008
Race				<0.001
White	464 (77%)	311 (83%)	153 (67%)	
Black	106 (18%)	43 (11%)	63 (28%)	
Other	33 (5%)	21 (6%)	12 (5%)	
Age (years)	49.7 ± 12.7	50.6 ± 13.2	48.3 ± 11.8	0.028
BMI (kg/m^2)	30.2 ± 6.8	30.7 ± 7.0	29.4 ± 6.4	0.023
SBP (mmHg)	127.9 ± 17.6	128.5 ± 18.1	127.1 ± 16.8	0.349
DBP (mmHg)	78.6 ± 11.4	78.1 ± 11.2	79.4 ± 11.8	0.178
Hypertension	247 (41%)	156 (42%)	91 (40%)	0.683
Diabetes	116 (19%)	65 (17%)	51 (22%)	0.128
MI	36 (6%)	24 (6%)	12 (5%)	0.632
Medication Use				
ASA	67 (12%)	46 (13%)	21 (10%)	0.295
Statin	82 (14%)	60 (17%)	22 (10%)	0.037
ACE or ARB	87 (15%)	63 (18%)	24 (11%)	0.047
Beta Blockers	53 (9%)	35 (10%)	18 (9%)	0.624

Values represent mean ± S.D. for continuous variables, and frequency (%) for categorical variables. P-values are based on t-test for continuous variables, and Chi-squared test for categorical variables. P-values represent the difference in demographic variables between the two groups: non-smokers and smokers. Abbreviations: BMI indicates body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MI, myocardial infarction; ASA, aspirin;

ACE, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker. Data in this table has been published in *Toxicology and Applied Pharmacology* (1).

Table 4. Summary Statistics of urinary VOC metabolites and Parent Compound for Circulating Angiogenic Cell analyses

Parent Structure	VOC	Metabolite	Non-Smokers Mean ± SD	n (%) LOD	Smokers Mean ± SD	n (%) LOD
	Acrolein	CEMA	115.8 ± 117.0	42 (11.2%)	338.6 ± 286.0	7 (3.1%)
		3HPMA	341.5 ± 344.6	0 (0%)	1763.7 ± 1594.1	0 (0%)
	Acrylamide	AAMA	72.4 ± 61.6	1 (0.3%)	171.5 ± 161.2	0 (0%)
	Acrylonitrile	CYMA	5.7 ± 17.2	231 (61.6%)	151.8 ± 137.4	7 (3.1%)
	Benzene	MU	133.4 ± 214.0	6 (1.6%)	166.8 ± 159.5	4 (1.8%)
	1,3-Butadiene	DHBMA	353.6 ± 175.9	0 (0%)	491.2 ± 246.2	0 (0%)
		MHBMA3	9.1 ± 8.2	18 (4.8%)	60.8 ± 49.3	1 (0.4%)
	Crotonaldehyde	HPMMA	300.0 ± 255.2	1 (0.3%)	1223.9 ± 789.2	0 (0%)
	N,N-Dimethylformamide	AMCC	160.4 ± 117.3	5 (1.3%)	527.5 ± 354.3	0 (0%)
	Ethylbenzene, styrene	PGA	225.9 ± 145.5	11 (2.9%)	428.9 ± 254.6	3 (1.3%)
	Propylene oxide	2HPMA	58.4 ± 122.9	4 (1.1%)	123.3 ± 555.4	1 (0.4%)
	Styrene, ethylbenzene	PHEMA	2.2 ± 4.1	174 (46.4%)	3.9± 4.0	37 (16.2%)
		MA	232.8 ± 165.5	1 (0.3%)	470.4 ± 299.9	0 (0%)
	Toluene	BMA	11.5 ± 12.1	4 (1.1%)	11.9 ± 12.9	0 (0%)
	Xylene	2MHA	30.1 ± 53.4	53 (14.1%)	95.0 ± 109.5	6 (2.6%)
		3MHA+4MHA	194.9 ± 303.1	30 (8.0%)	750.9 ± 653.4	3 (1.3%)

VOC metabolites were measured in 375 non-smoking participants, 25-70 years of age with low to severe CVD risk. IQR, interquartile range; LOD, limit of detection. Metabolite units: µg/g creatinine. The full chemical name and LOD for each metabolite is listed in *Supplemental Table 1.* Data in this table has been published in *Toxicology and Applied Pharmacology* (1).

The urinary metabolite of benzene (MU) was positively associated with CAC-10 and the urinary metabolite of toluene (BMA) was positively associated with CAC-5. Of the CAC subpopulations measured, CAC-3 was associated with the most urinary VOC

metabolites. The significant associations between CAC-3 and urinary metabolites of acrylonitrile (CYMA), styrene (PHEMA), and xylene (2MHA and 3MHA+4MHA) may suggest that CAC-3 is a sensitive biomarker to tobacco derived VOCs.

To examine the effect of ambient VOC exposure on circulating angiogenic cells without the confounder of tobacco smoke, we focused our subsequent analyses on the subgroup of non-smokers (Figure 5B, 5C). Overall, the analysis of non-smokers revealed similar associations to the analysis of total participants (Figure 5A). In non-smokers, the strongest effects were observed in PGA, which had significant inverse associations with CACs-1,2,3,4,5,6,7,13, and 15 (Figure 5C). Several other VOC metabolites were inversely associated with levels of CACs, including metabolites of acrylonitrile (CYMA), 1,3-butadiene (MHBMA3), and xylene (2MHA and 3MHA+4MHA). For benzene metabolite (MU), we found significant inverse associations with CAC-1 and its subpopulation CAC-7, along with a positive association with CAC-10. Positive associations were observed between the metabolite of toluene (BMA) and CAC-5, 11, 13, and 14, all of which share the $AC133^{+}$ marker. After correcting for multiple testing, PGA upheld significant inverse associations with CAC-1, 3, 5, 6, and 15 (Figure 5B). Furthermore, the urinary metabolite of benzene (MU) remained positively associated with CAC-10 and the toluene metabolite (BMA) remained positively associated with CAC-5. Overall, these data suggest that exposure to various VOCs is generally associated with lower levels of several CAC subpopulations in peripheral blood, notably the early progenitor CACs.

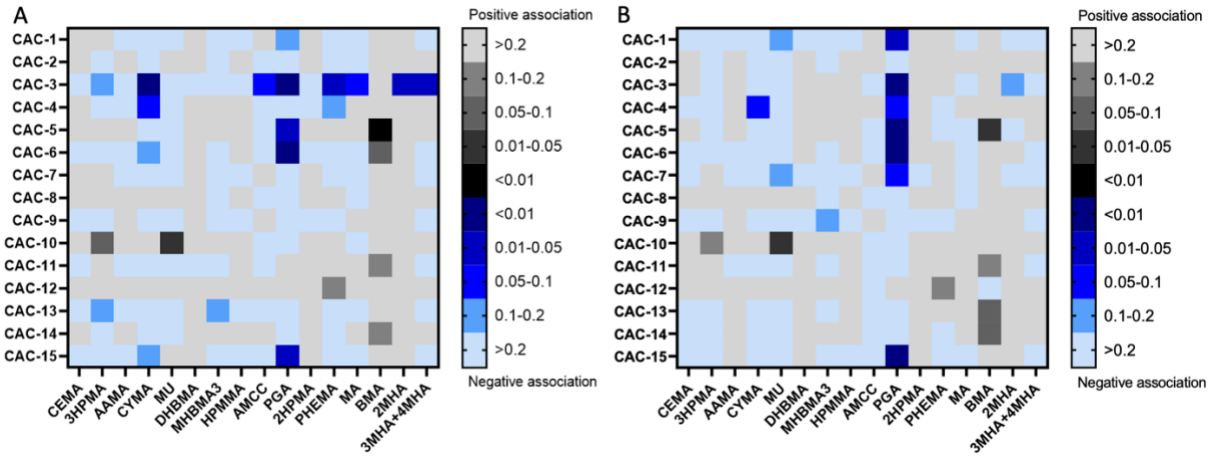


Figure 5. Heat map of pair-wise associations between the urinary metabolites of VOCs and circulating angiogenic cells. (A) False Discovery rate adjusted p-values for total sample (n=603). (B) False Discovery rate adjusted p-values for non-smokers (n=375). Generalized linear models were adjusted for age, sex, race, BMI, diabetes, hypertension, daily ozone, and daily $PM_{2.5}$ levels. Total samples was additionally adjusted for smoking status. Black grids represent positive associations and blue grids represent negative associations. The intensity of colors represent p-values, with darker grids indicating smaller p-values. This data has been published in *Toxicology and Applied Pharmacology* (1).

To determine the magnitude by which total VOC exposure can influence CAC levels and the directionality of the associations, we generated an Environmental Risk Score (ERS) for each circulating angiogenic cell population (Figure 6). We observed a strong negative risk score for CAC-1 (-62.9), CAC-3 (-54.6), and CAC-7 (-62.1), which all share common markers $CD45^{dim}/CD146^{+}/CD34^{+}$. We also found, to a lesser extent, negative risk scores for two $CD34^{+}$ CAC subpopulations, CAC-6 (-47.6) and CAC-9 (-37.7). In contrast, we found a positive risk score for the $CD45^{+}$ cell population CAC-12, as well as CAC-2, CAC-8, and CAC-14, which are subgroups of $CD45^{+}$ cells. These data indicate that levels of $CD45^{+}$ cells are positively associated with urinary metabolites of VOCs, whereas $CD45^{dim}$ and $CD34^{+}$ cells are negatively associated with urinary VOC metabolites.

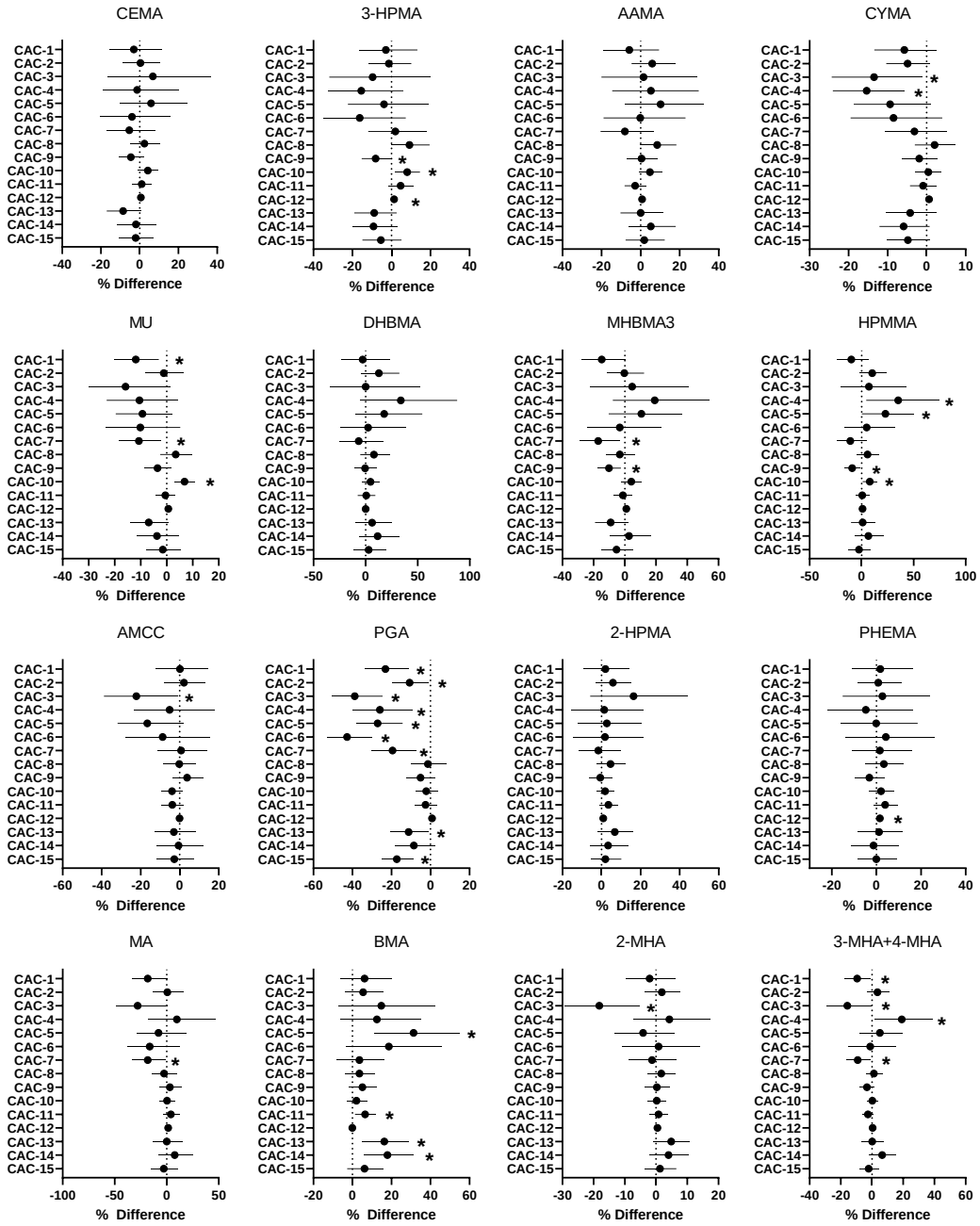


Figure 5. cont. (C) The X axis represents % difference (± 95% confidence interval) per 2-fold increase in VOC metabolite for each CAC sub-population (CAC 1-15) in non-smokers (n=375). Significant associations are shown in red. Generalized linear models were adjusted for age, sex, race, BMI, diabetes, hypertension, and daily $PM_{2.5}$ levels. Models for the full sample were additionally adjusted for smoking status.
Abbreviations: CEMA, N-Acetyl-S-(2-carboxyethyl)-L-cysteine; 3-HPMA, N-Acetyl-S-(3-hydroxypropyl)-L-cysteine; AAMA, N-Acetyl-S-(2-carbamoylethyl)-L-cysteine; CYMA, N-Acetyl-S-(2-cyanoethyl)-L-cysteine; MU, *trans, trans*-muconic acid; DHBMA, N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine; MHBMA3, N-Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine; HPMMA, N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine; AMCC, N-Acetyl-S-(N-methylcarbamoyl)-L-cysteine; PGA, Phenylglyoxylic acid ; 2HPMA, N-Acetyl-S-(2-hydroxypropyl)-L-cysteine; PHEMA,

N-Acetyl-S-(1-phenyl-2-hydroxyethyl)-L-cysteine +; MA, Mandelic Acid; BMA, N-Acetyl-S-(benzyl)-L-cysteine; 2MHA, 2-Methylhippuric acid; 3MHA+4MHA, 3-Methylhippuric acid + 4-Methylhippuric acid. This data has been published in *Toxicology and Applied Pharmacology* (1).

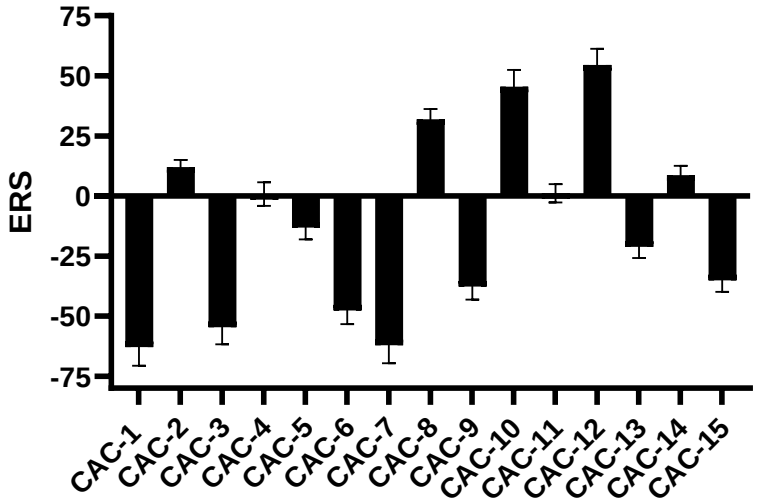
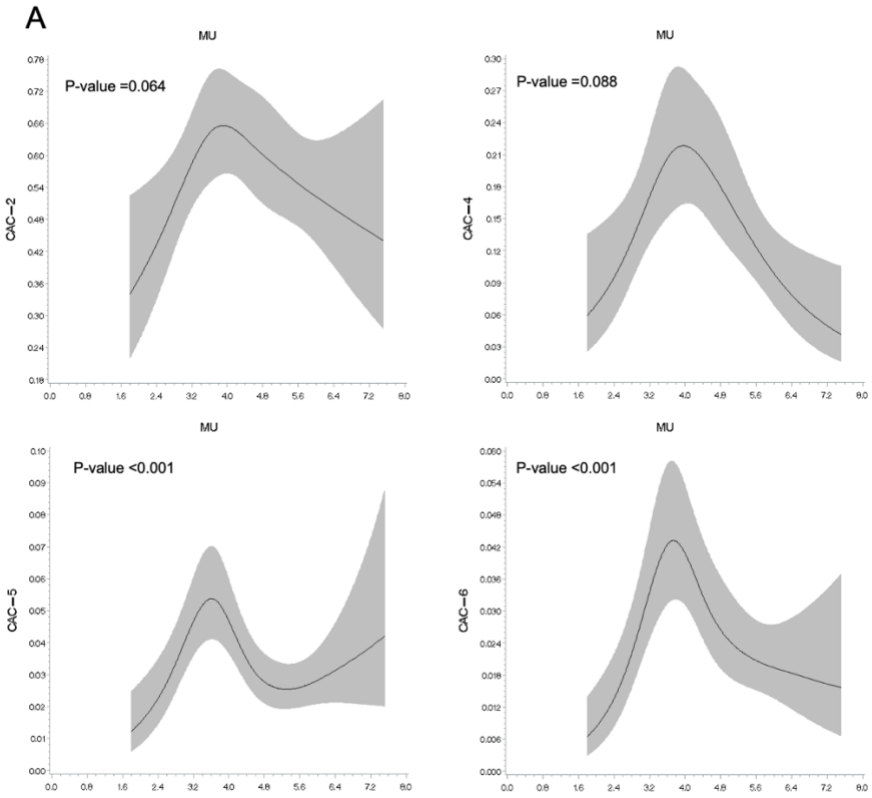


Figure 6. Association between Environmental Risk Scores and Circulating Angiogenic Cells in non-smokers. Environmental Risk score was calculated for each cell population as follows: 1. The urinary levels of VOC metabolites were log transformed. 2. Each log transformed VOC metabolite level was regressed against the levels of the cells separately, while adjusting for covariates. 3. ERS was calculated as the sum of the log transformed VOC metabolite levels x (regression coefficient/standard error). Mean and standard deviation of the ERS was calculated for each CAC. Values are mean ± SD of environmental risk score for each cell population. This data has been published in *Toxicology and Applied Pharmacology* (1).

Non-linear relationships were observed for associations between circulating angiogenic cells and urinary metabolites of VOCs. To evaluate potential non-linear relationships between CACs and VOC metabolites, we examined associations using restricted cubic splines (Figure 7). We found non-linear relationships between the levels of several circulating angiogenic cell populations and the metabolite of benzene (MU). Low levels of MU were associated with higher levels of circulating angiogenic cells, while higher levels of MU were associated with lower abundance of CACs (Figure 7A). For MU, near significant ($p<0.1$) non-linear relationships were seen with $CD146^{+}$ cell populations (CAC-2, 4, 5, and 6), and with AC133+ cell populations CAC-4 and CAC-5.

Next, we examined the associations of $CD146^+$ cells, CAC-4 ($CD45^+/CD146^+/AC133^+$) and CAC-8 ($CD45^+/CD146^+/AC133^-$), with VOC metabolites to see if $AC133^+$ cells contribute to the non-linear relationships (Figure 7B, 7C). We observed near significant ($p<0.1$) non-linear relationships between CAC-4 and the metabolites of acrolein (3HPMA), 1,3-butadiene (MHBMA3), N,N-dimethylformamide (AMCC), propylene oxide (2HPMA), styrene/ethylbenzene (MA), and xylene (3MHA+4MHA). For CAC-8, we did not observe the same non-linear relationships as we did for CAC-4, with the exception of xylene (3MHA+4MHA). These data suggest that early endothelial progenitor cells ($CD45^+/CD146^+/AC133^+$) are recruited at low levels of VOC exposure and depleted at high levels of VOC exposure.



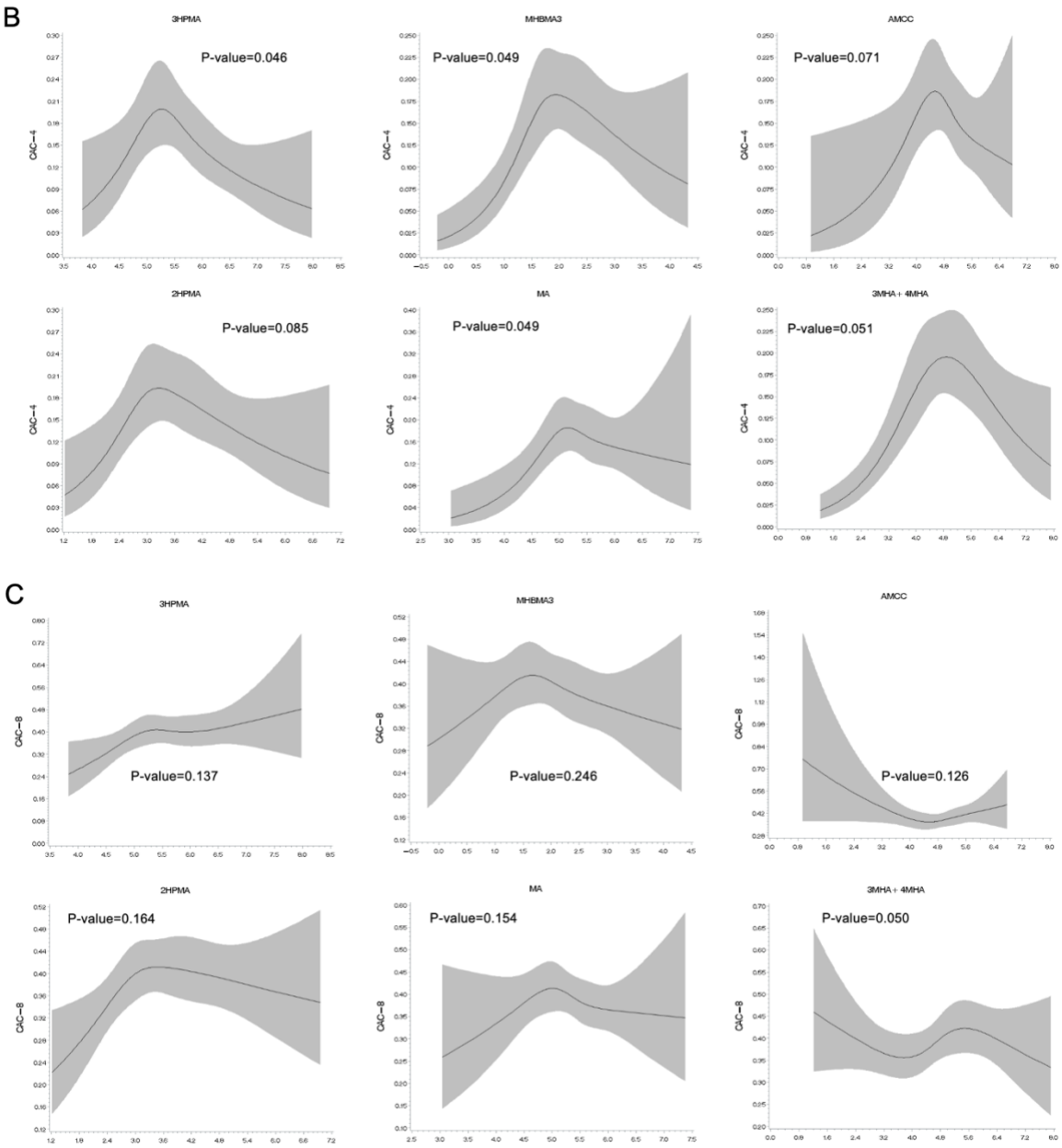


Figure 7. Exposure-response relationships between selected CACs and VOCs in non-smokers. Non-linear relationships between MU metabolites and CACs (A); VOCs and CAC-4 (**B**); and VOCs and CAC-8 (C) were examined using restricted cubic splines. Tests for non-linearity used the likelihood ratio test. Models are adjusted for age, sex, race, BMI, diabetes, hypertension, beta-blocker use, and daily $PM_{2.5}$ levels. P-value represents likelihood ratio test for non-linearity. This data has been published in *Toxicology and Applied Pharmacology* (1).

Associations between CACs and urinary VOC metabolites were influenced by participant demographic and cardiovascular disease risk factors. Since the metabolites of benzene, 1,3-butadiene, N,N-dimethylformamide, and ethylbenzene/styrene showed strong associations with CACs consistently, we performed

subgroup analyses to see if these associations were modified by participant demographic characteristics or their cardiovascular risk factors (Figure 8). We were able to see a variety of significant interactions among the relationships between urinary VOC metabolites and different CAC populations. For example, we found negative associations between CAC-3 and VOC metabolites (MU, AMCC, and PGA) in White participants, whereas Black participants had significantly higher levels of CAC-3. We also observed similar trends in the associations of CAC-13 and metabolites of benzene (MU) and ethylbenzene/styrene (PGA), in which Black participants had higher levels of CAC-13 compared to White participants.

We also found some evidence that suggest the relationships between VOC metabolites and CAC levels are modified by age and BMI. Aged participants (>55 years) showed stronger positive associations between CAC-5 and VOC metabolites compared to younger participants (<55 years). Older participants also had a strong positive association between CAC-7 and the urinary metabolite of N,N-dimethylformamide (AMCC). Although the directionality of BMI as a modifier was not consistent, we observed positive associations between CAC-3 and VOC metabolites (MU, AMCC) in participants with high BMI measurements (>30).

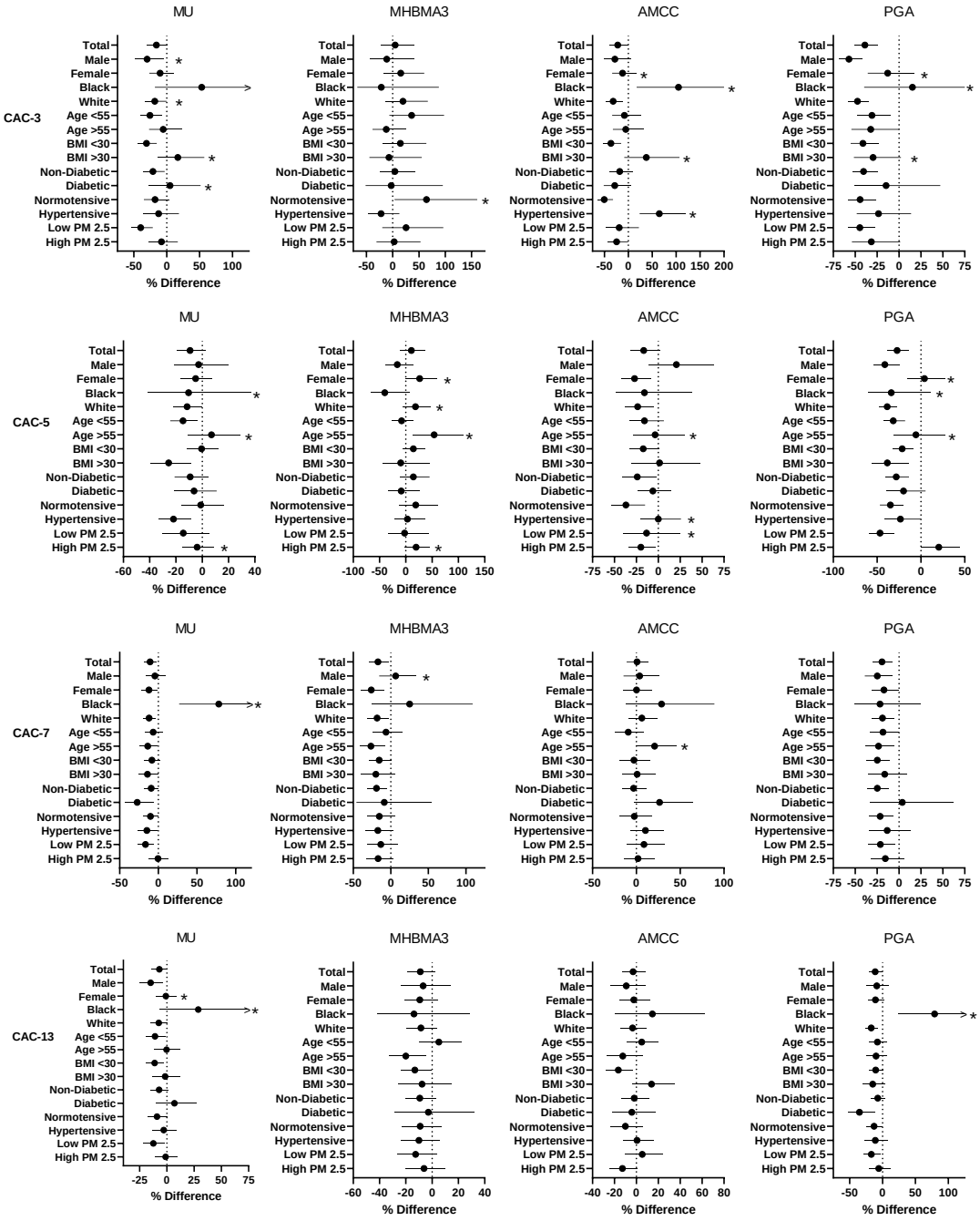


Figure 8. Subgroup analysis of the relationship between different CAC population with the urinary metabolites of benzene (MU), 1,3-butadiene (MHBMA3), ethylbenzene/styrene (PGA), and N,N-Dimethylformamide (AMCC) in non-smokers. Generalized linear models were adjusted for covariates - age, sex, race, BMI, diabetes, hypertension, and daily $PM_{2.5}$ levels, unless the covariates were used for stratification. Significant interaction between subgroups in the full model are indicated by an asterisk. This data has been published in *Toxicology and Applied Pharmacology* (1).

Next, we examined the influence of two risk factors which contribute to endothelial dysfunction, hypertension, and diabetes. Interestingly, we found stronger inverse associations between CACs and VOC metabolites in normotensive participants than hypertensive participants and, to a lesser extent, non-diabetics than diabetics. For both CAC-3 and CAC-5, we found significant inverse associations with metabolites of N,N-dimethylformamide (AMCC) and ethylbenzene/styrene (PGA) in normotensives, while the relationships in hypertensives were either not significant or positively associated. We also found several inverse associations in non-diabetics, whereas diabetic participants had higher percent differences per 2-fold increase in VOC metabolites. Overall, these data suggest that different cardiovascular risk factors may modify the response of circulating angiogenic cells in the context of VOC exposures.

DISCUSSION

The major findings of my studies were that urinary metabolites of environmental VOCs such as benzene, ethylbenzene, styrene, xylene, acrylamide, and 1,3-butadiene are positively associated with endothelial cell/CAC (aka EPC)-derived circulating microparticles; and metabolites of ethylbenzene/styrene, benzene, xylene, and 1,3-butadiene were negatively associated with CACs. For some VOCs, such as benzene, the low levels of their metabolites were positively associated with an increase in CAC levels, whereas high levels of exposure were associated with a significant decrease in CAC levels. Our environmental risk score recognized that while all the MPs measured were sensitive to VOCs, CAC-1, CAC-3, and CAC-7, with common cell markers $CD45^{dim}/CD146^{+}/CD34^{+}$, were the most sensitive to total VOC exposure. We also observed that sex, race, age, and hypertension were effect modifiers of some of the relationships between the levels of VOC metabolites with endothelial cell/EPC-derived MPs and CACs. Collectively, these findings suggest that exposure to VOCs could lead to endothelial activation/injury and deficits in endothelial repair capacity, which vary with sex, race, and comorbidities. Given that high endothelial-cell-derived MP and low CAC levels are predictive of future cardiovascular events and death, these results support the notion that VOC exposure is associated with significant endothelial injury, and it may be an important environmental risk factor for CVD. Although little is known about the vascular toxicity of VOCs in humans, results of this study complement experimental animal data demonstrating that exposure to benzene or its active metabolite (trans,trans

muconaldehyde) promote endothelial microparticle formation (94); and benzene (18) and acrolein exposure deplete EPCs (17).

Automobile emissions, industrial facilities, hazardous waste sites, and household products are major sources of VOC emissions, and therefore, VOC exposures are common and frequent (46). VOCs are also abundant in cigarette smoke. In our study, we found significant levels of VOC metabolites in the urine of non-smokers, indicating particularly high levels of exposure to acrolein, crotonaldehyde, 1,3-butadiene, and acrylamide, as well as the BTEXS (benzene, toluene, ethylbenzene, xylene and styrene) group of VOCs, suggesting a contribution of both indoor and outdoor exposure. Specifically, we found that the endothelial cell-derived MPs and CAC levels were particularly sensitive to metabolites of BTEXS compounds.

Although exposure to BTEXS is common, little is known about their cardiovascular effects. A study from Hong Kong found that short term elevations in atmospheric levels of benzene and TEX (toluene, ethylbenzene, and xylene) was associated with increases in circulatory disease mortality by 5.8% and 3.5% (95), although in a study from Toronto, no intra-urban variations in VOC levels were found be associated with cardiovascular disease mortality (16). BTEXS such as toluene, styrene, ethylbenzene, and xylenes have also been linked to a higher prevalence of CVD (96). Additionally, exposure to benzene, ethylbenzene, and styrene have been found to be associated with higher odds of developing metabolic syndrome (97). Recent studies have also suggested that ambient VOC exposure, especially alkyne and benzene, is associated with increased risk for heart failure (12). In experimental animals, chronic benzene exposure exacerbates cardiac dysfunction (98). Along with these data, the results of this study support the notion that exposure to VOCs could have adverse cardiovascular consequences, potentially due to low-level vascular injury reflected by changes in MPs and CAC levels.

My data indicates positive associations between MPs from all cell types measured and several urinary VOC metabolites, with the most significant associations in EPC MPs, endothelial MPs, and activated endothelial MPs. Environmental Risk Scores were created to visualize the extent to which VOC exposure influences the different MPs. Strong positive risk scores (>150) were observed for all MPs measured, indicating that levels of MPs in circulation are positively associated with urinary metabolites of VOCs. Similar observations were made with pair-wise associations between individual VOC metabolites and MP subtypes.

Although the direct effect of VOCs on vascular function and toxicity are unclear, the endothelium, which is a regulator of vascular homeostasis, vascular tone, thrombosis, and angiogenesis, is particularly vulnerable to the effects of tobacco smoke which contains high levels of VOCs. In smokers, endothelial dysfunction is the earliest sign of injury (99). To examine the effect of VOC exposure on endothelial changes, we measured the levels of microparticles. Increased endothelial microparticles in the blood correlate with endothelial dysfunction in patients with coronary artery disease (70), type 2 diabetes (55, 100), and obesity (101). My observations that endothelial MPs are positively associated with metabolites of benzene, ethylbenzene, styrene, and xylene suggest that exposure to gasoline products could increase endothelial toxicity. Moreover, because endothelial MPs express Von Willebrand factor and factor VIII which stimulate platelet activation (102, 103), VOC-induced endothelial MP formation could also promote platelet activation, thrombosis, and subsequent cardiovascular events. My studies complement animal studies data demonstrating that chronic benzene exposure increases circulating endothelial microparticles (94) and platelet activation, and *in vitro* studies revealing that benzene metabolite trans, trans muconaldehyde facilitates endothelial MP formation from apoptotic aortic endothelial cells (94). It is likely that people living near gas stations, gasoline refineries, and working with aromatic

hydrocarbons are at increased risk of endothelial damage, compromised endothelial function, and potentially cardiovascular disease. Moreover, landfills containing industrial waste may also contain high levels of aromatic hydrocarbons. Excessive rates of type 2 diabetes (T2D) and stroke have been found in an evaluation of 720,000 individuals living within a half-mile of 258 Superfund sites that were associated with excessive VOC (such as benzene) in the drinking water (104). These rates correspond to roughly 8,600 excessive T2D cases and 8,600 excessive stroke cases with an estimated total annual cost of $160 million for long-term care and lost productivity (104).

The positive association between the metabolite of acrylamide and endothelial MPs suggests that cigarette smoke-induced endothelial toxicity could, at least in part, be attributed to acrylamide. Because acrylamide is also abundant in food cooked at high temperatures such as potato products (e.g., French fries and potato chips), food made from grains (e.g., cookies and toast), and coffee (105), increased consumption of these foods may also increase the risk of endothelial damage.

My studies also reveal that activated endothelial MPs are positively associated with the metabolites of crotonaldehyde, N,N-dimethylformamide, ethylbenzene, and xylene. The positive association of activated lung endothelial MPs with VOCs (e.g. benzene, N,N-dimethylformamide, ethylbenzene, and propylene oxide) suggest that the pulmonary endothelium might be the first target of inhaled VOCs. Previous studies have shown that increased circulating lung endothelial MPs in healthy smokers precede changes in pulmonary function (106). Therefore, it is conceivable that VOC exposure-induced pulmonary endothelial damage may lead to compromised pulmonary functions.

Like endothelial MPs and activated endothelial MPs, EPC MPs, were positively associated with xylene (2MHA). The consistent positive associations with xylene observed among endothelial MPs, activated endothelial MPs, and EPC MPs suggests that exposure to xylene is intimately related to endothelial activation and injury.

Enhanced circulating activated endothelial microparticles in the blood may increase cardiovascular events (107). Because petroleum products and cigarette smoke contain high levels of the VOCs associated with increased activated endothelial microparticles, smoking and increased occupational, environmental, and residential exposure to these VOCs could augment the formation of activated endothelial MPs and increase cardiovascular events risk.

My findings also demonstrate significant interactions between MPs and urinary VOC metabolites for sex-dependent, age-dependent, and BMI-dependent subgroups. Female participants consistently showed higher levels of 1,3-butadiene (DHBMA) urinary metabolite in relation to EPC, endothelial, activated endothelial, and lung MPs compared to male participants, who had the most significant interaction between toluene (BMA) urinary metabolite and those same microparticle subtypes. These data suggest that females are likely to be at increased risk for 1,3-butadiene-induced endothelial injury whereas men may be more prone to toluene-induced endothelial toxicity.

Stratification of data by age revealed that participants over the age of 55 showed a higher percentage of difference for all comparisons. Specifically, 1,3 butadiene metabolite had significant positive interactions for all microparticle subtypes measured in participants over the age of 55, compared to participants younger than 55 years old. This suggests a greater vulnerability for older individuals to the cardiovascular toxicity of VOCs. Results for BMI-dependent relationships were mixed, which may be attributed to the lipophilic nature of many VOCs, and therefore the urinary VOC levels may not fully represent the exposure in obese individuals.

An abundance of microparticles in circulation is reflective of cellular damage. As an adaptive response, CACs are mobilized from the bone marrow to facilitate endothelial repair and/or neovascularization. In contrast to microparticles, quantities of CACs are reduced in patients with cardiovascular and inflammatory diseases, including coronary

artery disease (70), rheumatoid arthritis (76-78), and systemic lupus erythematosus (79, 80). Low numbers of CACs have also been demonstrated to predict cardiovascular events (i.e., myocardial infarction, unstable angina, ischemic stroke, and death) and atherosclerotic disease progression (70, 108). Some studies have utilized both circulating angiogenic cells and microparticles as surrogate biomarkers in parallel to gain a more comprehensive insight into cardiovascular health status. In a study by *Pirro et al.*, hypercholesterolemic patients exhibited higher levels of endothelial MPs ($CD31^+/CD42^-$), lower levels of CACs ($CD34^+/KDR^+$), and increased aortic stiffness compared to control individuals (109). Additionally, they found that an increased ratio of MPs to CACs was a predictor of increased aortic stiffness, which reflects an imbalance between endothelial damage and repair. Given that CACs have gained traction as indicators of cardiovascular health, we measured associations between 15 phenotypically distinct CAC populations and urinary metabolites of 12 VOCs to examine relationships between VOC exposure and CVD risk.

My data reveal that the levels of circulating angiogenic cells (CACs) in peripheral blood were significantly associated, most negative and some positive, with the levels of several urinary VOC metabolites in single-VOC analyses. For all participants, the results showed negative associations between PGA (ethylbenzene or styrene metabolite) and CAC-3, 5, 6, and 15. Positive associations were observed between the urinary metabolite of benzene (MU) and CAC-10, and between the urinary metabolite of toluene (BMA) and CAC-5. CAC-3 was associated with the most urinary metabolites and showed significant associations with metabolites of acrylonitrile, styrene, and xylene, suggesting that CAC-3 is a sensitive biomarker to tobacco-derived VOCs. To examine the effects of ambient VOC exposure on CACs without the confounder of tobacco smoke, a non-smokers subgroup analysis was conducted and revealed associations similar to the total group.

For the subgroup of non-smokers, an Environmental Risk Score (ERS) was generated for each CAC subpopulation. The ERS analysis revealed that CAC-1, CAC-3, and CAC-7, all of which share $CD45^{dim}/CD146^{+}/CD34^{+}$, were the most sensitive to total VOC exposure and had strong negative risk scores. In the opposite direction, strong positive risk scores were observed for CAC-8 ($CD45^{+}/CD146^{+}/AC133^{-}$), CAC-10 ($CD146^{+}$), and CAC-12 ($CD45^{+}$). Additionally, $CD45^{+}$ populations CAC-2 and CAC-14 showed positive risk scores to a lesser extent. By generating Environmental Risk Scores, we were able to observe the directionalities of each CAC in relation to total VOC exposure. Moreover, evaluation of non-linear relationships indicated that early progenitor cells ($CD45^{+}/CD146^{+}/AC133^{+}$) are recruited at low levels of VOC exposure and depleted at high levels of exposure in a biphasic response. These data suggest that VOC exposure is associated with significant vascular injury and repair independent of tobacco use, and therefore it may be a risk factor for cardiovascular disease.

In further subgroup analyses, covariates such as sex, race, age, body-mass index, and hypertension were observed to be effect modifiers of some relationships between CACs and the levels of VOC metabolites. Black participants had greater extent of negative associations between CAC-3 and benzene (MU), N,N-dimethylformamide (AMCC), and ethylbenzene (PGA) metabolites and between CAC-12 and benzene (MU) and ethylbenzene (PGA) metabolites compared to white participants. Participants over the age of 55 showed stronger positive association between CAC-5 and VOC metabolites and between CAC-7 and AMCC metabolite compared to younger participants. BMI subgroup comparisons were not consistent, with the relationships between CAC-3 and benzene (MU) and N,N-dimethylformamide (AMCC) metabolites showing a greater positive association for participants with BMI greater than 30. Stronger inverse associations between CACs and VOC metabolites were observed in normotensive participants than hypertensive participants, and to a lesser extent in non-

diabetics than diabetics. For CAC-3 and CAC-5, significant inverse associations with N,N-dimethylformamide (AMCC) and ethylbenzene (PGA) metabolites were observed in normotensive participants while the hypertensive subgroup showed positively associated or not significant. These data suggest that different covariates and cardiovascular risk factors may modify the response of particular CACs in the context of VOC exposure.

Given the global burden of cardiovascular disease and ubiquitous distribution of volatile organic compounds, it is crucial to assess the implications of ambient VOC exposure on human health. In this study, we examined many associations between exposure and measures of preclinical cardiovascular injury (MPs & CACs) which are predictive of CVD and mortality. Overall, levels of VOC metabolites were positively associated with circulating microparticles (MPs) and negatively associated with circulating angiogenic cells (CACs). A major strength of this study is the use of individual level data to precisely analyze relationships between VOC exposure and subclinical biomarkers of cardiovascular injury. Although measurements of urinary VOC metabolites do not indicate the sources or routes of exposure, they do provide a high throughput and integrated measure of an individual's total exposure. The findings of this study highlight the potential dangers of VOC exposure and the need for heightened awareness of such exposures that may otherwise go unnoticed.

Our study also has some limitations. Because of the cross-sectional nature of the study, the associations found in this study cannot address causality. Longitudinal studies are needed to determine whether VOC exposures are associated with adverse clinical outcomes, and whether these outcomes are related to changes in MPs and CAC levels. Moreover, we used spot urine collection from each individual to estimate VOC exposure. Although, it has been reported that the inter-day reproducibility for most urinary VOC metabolites is good to excellent (110, 111). Following exposure, many VOCs rapidly undergo biotransformation to generate metabolites that can conjugate with glutathione.

Other enzymatic reactions remove glutamic acid and glycine to produce a cysteine conjugate, which is *N*-acetylated and excreted in the urine as mercapturic acid (46). It has also been shown that smoking one cigarette, which generates high levels of VOCs, is sufficient to immediately affect the levels of CACs (112). Therefore, because VOC metabolites and CAC levels were concurrently measured, it is likely that the observed associations represent consequences of recent exposure. Additionally, our urinary VOC metabolites were normalized to creatinine to account for dilution differences, which could introduce error that relates to the dependence of creatinine excretions due to muscle mass, physical activity, and kidney disease. Another limitation of our study is that most participants were, by study design, of older age and at increased risk of cardiovascular disease. Thus, our sample could represent a group of susceptible individuals, which may limit generalizability of our findings.

SUMMARY

Volatile organic compounds (VOCs) are reactive chemicals that are readily emitted from certain solids and liquids. Many VOCs are human-made chemicals which are used and generated from anthropogenic sources, including petrochemical processing, industrial solvents, and the production of rubber and plastics. VOCs are also highly abundant at *Superfund & Hazardous Waste Sites* across the United States. Other sources of VOC exposure include automobile exhaust, tobacco smoke, cleaning supplies, and personal care products. Although VOCs can persist in groundwater, their volatile nature allows them to evaporate into the air at ambient temperatures. A variety of VOCs that pose a risk to human health have been identified in both indoor and outdoor air. Studies have shown that exposures to certain VOCs, like benzene and formaldehyde, are significantly associated with carcinogenicity and damage to vital organs, such as the liver, kidney, central nervous system. Potential cardiovascular effects of tobacco-derived VOCs have been studied, but the effects of ambient VOCs on cardiovascular health remains unclear. Furthermore, VOCs have been identified as constituents of air pollution, which is a significant contributor of cardiovascular disease (CVD) around the world.

In this cross-sectional study, we recruited over 600 participants with low-to-high CVD risk and measured the relationships between urinary biomarkers of VOC exposure and subclinical biomarkers of harm in peripheral blood. We quantified cell-derived microparticles (MPs) and circulating angiogenic cells (CACs) by flow cytometry to assess endothelial injury and endothelial repair capacity, respectively. Studies have illustrated a notable elevation of circulating MPs in patients with cardiovascular disease

(CVD) and CVD risk factors. Additionally, growing evidence suggests that elevated levels of circulating MPs may contribute to inflammation, endothelial dysfunction, atherosclerosis, and overall deterioration of cardiovascular health.

By and large, we observed that urinary metabolites of several VOCs were positively associated with circulating microparticles of various cellular origin. The positive pair-wise associations that we observed remained significant after adjusting for multiple covariates, including smoking status, age, sex, race, BMI, diabetes, hypertension, daily ozone, and daily $PM_{2.5}$ levels. We generated environmental risk score (ERS) that indicated strong positive risk scores for all of the MP types measured in non-smokers. These data solidify the notion that elevated MPs in circulation are associated with exposures to VOCs independent of other major risk factors for CVD. Overall, we observed that lung MPs, lung endothelial MPs, and activated lung endothelial MPs had higher numbers of positive associations with urinary VOC metabolites than endothelial MPs, activated endothelial MPs, and EPC MPs. This may indicate that VOC-induced pulmonary toxicity is primary to cardiovascular injury. We also found sex, age, and BMI to be modifiers of some relationships between the levels of VOC metabolites and MPs.

Alongside MP measurements as markers of damage, we also measured circulating angiogenic cells (CACs) which are reflective of endothelial repair capacity. We found that the urinary metabolites of several common VOCs, such as benzene, xylene, ethylbenzene/styrene, and 1,3-butadiene were negatively associated with CACs. Interestingly, for some VOCs like benzene, we observed that low levels of metabolites were positively associated with elevated CAC levels, while higher levels of metabolites were associated with a significant decline in CAC levels. The environmental risk score generated for each of the 15 CAC populations revealed the extent to which total VOC exposure affected CAC levels, as well as the directionality of the associations. CAC-1, CAC-3, and CAC-7, which share common cell markers $CD45^{dim}/CD146^{+}/CD34^{+}$, were

the most sensitive CAC populations and had negative risk scores. In comparison, we found positive risk scores for $CD45^+$ cells (CAC-12) and subgroups of $CD45^+$ cells (CAC-2, CAC-8, and CAC-14). The differing directionalities among CAC subpopulations may be explained by population-specific function, as well as a biphasic response we observed in our non-linear relationships. Our results suggest that exposure to VOCs could result in deficits in endothelial repair capacity in a dose-specific manner which may vary with sex, race, and comorbidities. Since low CAC levels are predictive of cardiovascular events and death, and our results indicate that VOC exposure is associated with vascular injury and CVD risk independent of tobacco smoke, VOCs should be considered significant environmental risk factors for CVD.

While the cardiovascular toxicity of VOCs from tobacco smoke has been studied extensively, the potential cardiovascular effects of ambient VOC exposure has not been highly emphasized. For both microparticle and circulating angiogenic cell measurements, we evaluated associations with VOC metabolites in the total group of participants and a subgroup of non-smokers. Interestingly, we found similar associations in both groups, suggesting that the effects of VOCs on MPs and CACs is independent of tobacco smoke exposure and environmentally low levels of VOCs may increase the risk of cardiovascular injury. Given that there are numerous sources of VOC emissions, including automobile exhaust, industrial facilities, hazardous waste sites, and even commonly used household products, VOC exposures are common and frequent. In our study, we found significantly high levels of VOC metabolites in the urine of non-smokers, which indicate high levels of exposure to VOCs such as acrolein, crotonaldehyde, 1,3-butadiene, acrylamide and BTEX compounds (benzene, toluene, ethylbenzene, xylene, and styrene). These findings suggest that VOC exposures are occurring in both indoor and outdoor settings. Specifically, we observed that the CAC levels were particularly

sensitive to BTEX compounds, while MPs were positively associated with a variety of urinary VOC metabolites.

In conclusion, our study demonstrates that microparticles and circulating angiogenic cells are significantly associated with urinary metabolites of VOCs, supporting the plausibility that VOC exposures contribute to cardiovascular disease progression and exacerbate pre-existing conditions. These subclinical biomarkers of harm may be useful tools in the clinical setting for assessing cardiovascular health before the occurrence of clinically significant events. For example, atherosclerosis is a slow and progressive disease that is characterized by endothelial damage/dysfunction, lesion formation, and plaque accumulation which leads to luminal narrowing of arteries and vessels. Circulating MPs may have prognostic value for identifying vascular and endothelial damage, while CACs may be used to assess vascular repair capacity. Moreover, the findings of this study emphasize the possibility that environmental exposure to VOCs may be an important underlying determinant of CVD risk. Although our cross-sectional study is insightful, longitudinal and mechanistic studies are needed to confirm our findings and gain more knowledge on how VOC exposures influence cardiovascular health. If our evidence is confirmed, associations between VOC exposure and CVD risk could be integrated into the estimates of CVD risk and recommendations could be proposed to limit both ambient and indoor VOC exposures.

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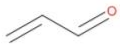
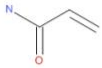
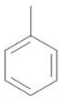
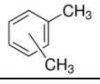
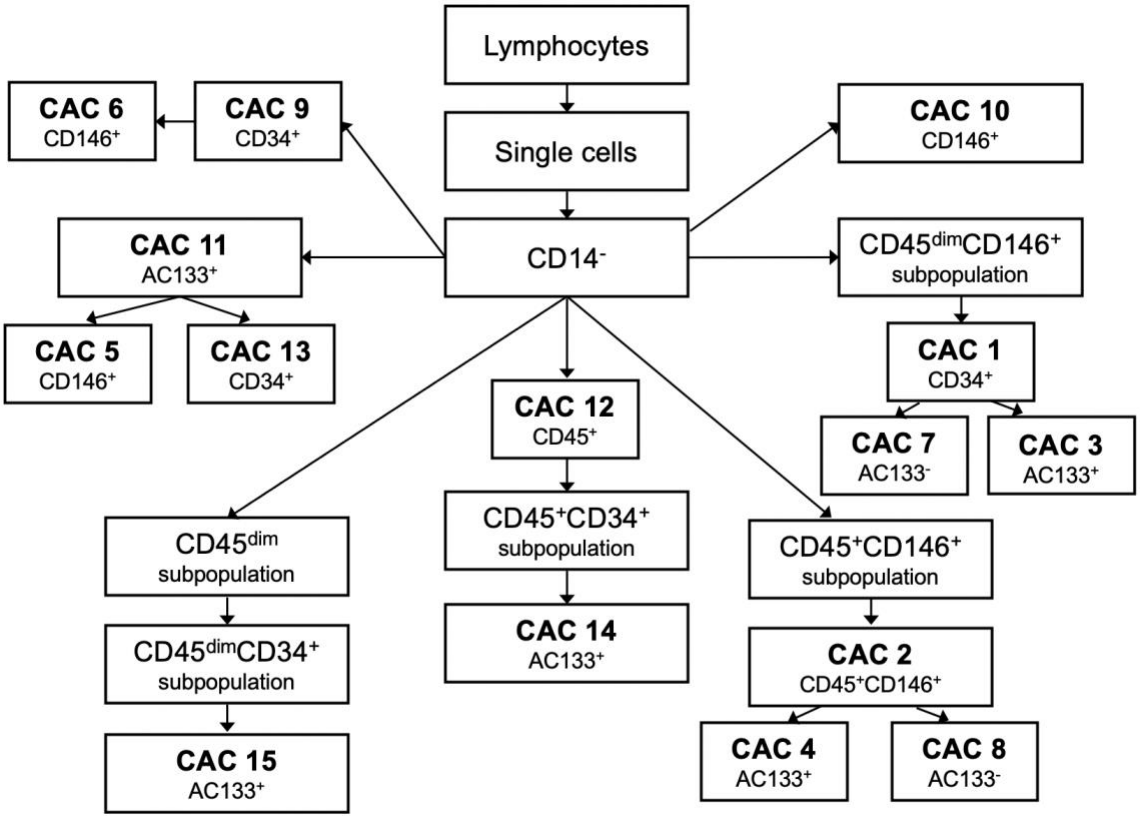
SUPPLEMENTARY MATERIAL

Supplemental Table 1. Volatile organic compounds and urinary metabolites.

Parent Structure	VOC	VOC Metabolite	Common Abbr.	LOD (ng/mL)
	Acrolein	N-Acetyl-S-(2-carboxyethyl)-L-cysteine	CEMA	20.35
		N-Acetyl-S-(3-hydroxypropyl)-L-cysteine	3HPMA	9.12
	Acrylamide	N-Acetyl-S-(2-carbamoylethyl)-L-cysteine	AAMA	3.03
	Acrylonitrile	N-Acetyl-S-(2-cyanoethyl)-L-cysteine	CYMA	1.25
	Benzene	Trans, trans-Muconic acid	MU	6.41
	1,3-Butadiene	N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine	DHBMA	6.76
		N-Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine	MHBMA3	1.44
	Crotonaldehyde	N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine	HPMMA	1.11
	N,N-Dimethylformamide	N-Acetyl-S-(N-methylcarbamoyl)-L-cysteine	AMCC	9.87
	Ethylbenzene, styrene	Phenylglyoxylic acid	PGA	6.86
	Propylene oxide	N-Acetyl-S-(2-hydroxypropyl)-L-cysteine	2HPMA	3.97
	Styrene, ethylbenzene	N-Acetyl-S-(1-phenyl-2-hydroxyethyl)-L-cysteine +	PHEMA	1.21
		Mandelic acid	MA	12.93
	Toluene	N-Acetyl-S-(benzyl)-L-cysteine	BMA	0.45
	Xylene	2-Methylhippuric acid	2MHA	1.24
		3-Methylhippuric acid + 4-Methylhippuric acid	3MHA+4MHA	6.85

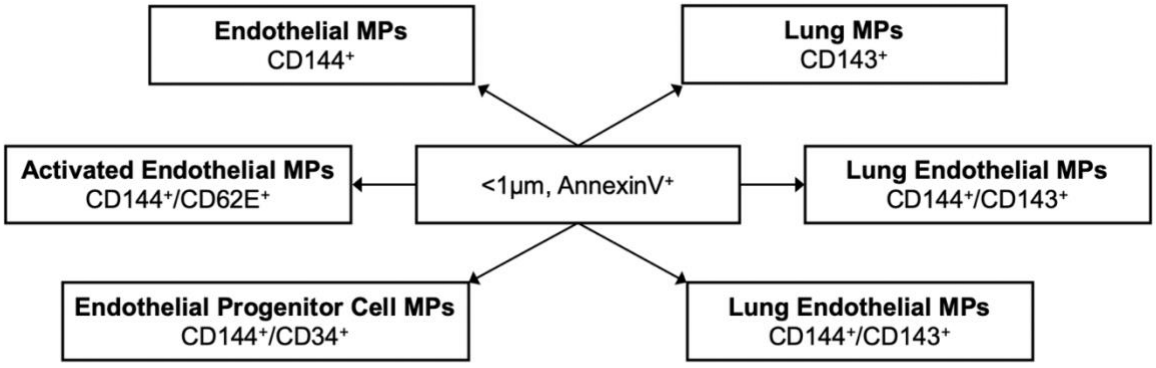
This table has been published in *Toxicology and Applied Pharmacology* (1).

Supplemental Figure 1. Classification of Circulating Angiogenic Cells.



This figure has been modified from a publication in *Toxicology and Applied Pharmacology* (1).

Supplemental Figure 2. Classification of cell derived Microparticles.



CURRICULUM VITAE

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Education

2013 – 2017	B.A., Chemistry, Biology minor University of Louisville, Louisville, KY
2019 – Present	M.S./Ph.D., Pharmacology & Toxicology University of Louisville School of Medicine, Louisville, KY

Research Experience

2017	Undergraduate Research Associate University of Louisville Department of Chemistry, Louisville, KY
2018 – 2019	Post-Baccalaureate Trainee/ Research Technician University of Louisville Christina Lee Brown Envirome Institute, Louisville, KY
2019 – Present	Graduate Trainee University of Louisville Department of Pharmacology and Toxicology, Louisville, KY

Other Experience and Professional Memberships

2019 – Present	Trainee, Superfund Research Program (SRP)
2019 – Present	Member, Christina Lee Brown Envirome Institute
2022 – Present	Trainee, National Institute of Environmental Health Sciences (NIEHS)
2022 – Present	Student/Postdoc/Alumni Network (SPAN) Representative with University of Louisville Superfund Research Center

Honors and Awards

2013 – 2017	Academic Commonwealth Scholarship
2014 – 2017	Morton Walker Scholarship
2019 – Present	Christina Lee Brown Envirome Institute Scholarship
2021 – 2022	NIEHS, K.C. Donnelly Externship Award
2022 – Present	NIEHS T32 Pre-doctoral Award, University of Louisville

Presentations

1. Poster presentation, B. Taylor, M. Malovichko, R.J. Keith, D. Riggs, A. Bhatnagar, S. Srivastava. “Volatile Organic Compound Exposure Depletes Circulating Angiogenic Cells”, NIEHS Superfund Research Program Annual Meeting, Seattle, WA, 2019.
2. Poster presentation, B. Taylor, N. Wickramasinghe, M. Malovichko, S. Sithu, S. Uchida, M. Nantz, S. Srivastava. “Contribution of Adenosine Deaminases Acting on RNA-1 (ADAR-1) in Benzene Metabolite t,t- Muconaldehyde-Induced Endothelial Activation”, NIEHS Superfund Research Program Annual Meeting, Virtual, 2020.

Publications

1. Publication, Malovichko MV, Abplanalp WT, McFall SA, Taylor BS, Wickramasinghe NS, Sithu ID, et al. Subclinical markers of cardiovascular toxicity of benzene inhalation in mice. Toxicology and Applied Pharmacology. 2021;431:115742.
2. Publication, Zelko IN, Taylor BS, Das TP, Watson WH, Sithu ID, Wahlang B, et al. Effect of vinyl chloride exposure on cardiometabolic toxicity. Environmental Toxicology. 2022;37(2):245-55.
3. Publication, Riggs DW, Malovichko MV, Gao H, McGraw KE, Taylor BS, Krivokhizhina T, et al. Environmental exposure to volatile organic compounds is associated with endothelial injury. Toxicology and Applied Pharmacology. 2022;437:115877.