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TOBACCO PRODUCT-DERIVED ALDEHYDES: EFFECTS ON CIRCULATING
ANGIOGENIC CELLS & IMPLICATIONS FOR CARDIOVASCULAR DISEASE

By

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B.S., McKendree University, 2011
M.S., Southern Illinois University Edwardsville, 2013
M.S., University of Louisville, 2016

A Dissertation
Submitted to the Faculty of the
School of Medicine of the University of Louisville
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for the Degree of

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in Pharmacology and Toxicology

Department of Pharmacology and Toxicology
University of Louisville
Louisville, KY

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DEDICATION

This dissertation is dedicated to Shelby and Lorene Lashley,
whose love and support helped make all my higher schooling possible.

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I would like to thank my mentor, Daniel J. Conklin, Ph.D., for all his advice and direction during my time in his lab. I would also like to thank my committee members for their insight and assistance. Additional thanks to Dr. Andrew DeFilippis, Karen Beatty, and Jessica Nystoriak for their advice and assistance in the Louisville Healthy Heart study; Gregg Shirk, Whitney Theis, and Lexi Hand for assistance with the murine exposures; and Dan Riggs for assistance on statistics. Thanks also to the National Institutes of Health for my funding (ES019217, GM127607, HL122676, HL149351, U54HL120163, T32ES011564).

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ABSTRACT

TOBACCO PRODUCT-DERIVED ALDEHYDES: EFFECTS ON CIRCULATING ANGIOGENIC CELLS & IMPLICATIONS FOR CARDIOVASCULAR DISEASE

Jordan B. Lynch

April 17, 2020

Cardiovascular disease (CVD) is the leading cause of death worldwide. Tobacco smoke is the most significant modifiable risk factor in CVD development and contains numerous toxic compounds, including aldehydes, which have been linked to CVD. Formaldehyde, acetaldehyde, acrolein, and crotonaldehyde have been identified as significant contributors to cigarette-induced health effects, but the mechanism of these effects is not well understood. These aldehydes are also found in aerosols produced by e-cigarettes (e-cigs). The goal of this dissertation was to explore the systemic, hematological, and endothelium-related effects of exposure to tobacco products and constituent aldehydes and to identify potential mechanisms of injury. In the Louisville Healthy Heart Study, hematological measures and circulating angiogenic cells (CACs) were assessed against tobacco product use categories. In murine studies, male and female C57BL/6J, transient receptor potential ankyrin 1 (TRPA1)-null, and/or extracellular superoxide dismutase-transgenic (ecSOD-Tg) mice were exposed to mainstream cigarette smoke (MCS), e-cig aerosol, humectants, or constituent aldehydes for varying durations and concentrations. Hematological measures, plasma biomarkers, and levels of CACs were assessed. Chapter III of this dissertation describes findings from the Louisville Healthy Heart Study. E-cig

or dual users showed significantly increased levels of leukocytes and of CAC-3, an early endothelial progenitor cell population, compared with never smokers. Chapter IV describes systemic and vascular changes induced by exposure to MCS, e-cig aerosol, or humectants, finding significant decreases in leukocytes after all exposures and decreases in CACs in female mice exposed to JUUL Mango aerosol. Chapter V describes effects of exposure to formaldehyde and acetaldehyde, finding that formaldehyde induces significant hematological and vascular changes. Chapter VI describes effects of exposure to acrolein and crotonaldehyde, presenting novel evidence implicating chronic crotonaldehyde exposure as a risk factor for CVD. Finally, chapter VII explores the roles of ecSOD and TRPA1 in tobacco product-related CVD, demonstrating that the effects of unsaturated aldehydes are mediated by TRPA1. This dissertation presents novel data investigating associations between CACs and e-cigs in a human cohort, examining the role of tobacco products and individual constituent aldehydes in the development of CVD, and demonstrating the role of TRPA1 in the CVD effects related to tobacco products.

TABLE OF CONTENTS

| | PAGE |
|---|------|
| DEDICATION | iii |
| ACKNOWLEDGEMENTS | iv |
| ABSTRACT | v |
| LIST OF TABLES | xii |
| LIST OF FIGURES | xvi |
| | |
| CHAPTER | |
| I. INTRODUCTION | 1 |
| A. Background and Rationale | 1 |
| 1. Cardiovascular Disease and Air Pollution | 1 |
| 2. Reactive Oxygen Species | 4 |
| 3. Circulating Angiogenic Cells | 5 |
| 4. Tobacco Smoke and Cardiovascular Disease | 6 |
| 5. Aldehydes | 7 |
| a. Formaldehyde and Acetaldehyde | 8 |
| b. Acrolein | 11 |
| c. Crotonaldehyde | 12 |
| 6. Electronic Cigarettes | 13 |
| 7. Transient Receptor Potential Ankyrin 1 Channel | 19 |
| 8. Statement of Goals | 20 |
| B. Aims and Proposals | 20 |

| | |
|---|----|
| 1. Measure Hematological and Endothelium-Related Changes in Humans in Response to Tobacco-Product Related Pollutant Exposures | 20 |
| 2. Measure Systemic, Hematological, and Endothelium-Related Changes in Mice in Response to Tobacco Products and Constituent Aldehydes | 21 |
| 3. Test the Roles of ecSOD and TRPA1 in the Development of Hematological, Systemic, and Endothelium-Related Changes Contributing to CVD | 21 |
| C. Overall Aims of this Dissertation | 22 |
| II. EXPERIMENTAL PROCEDURES | 24 |
| A. Human Studies | 24 |
| 1. Study Population | 24 |
| 2. Eligibility | 24 |
| 3. Case Definition | 25 |
| 4. Recruitment | 25 |
| 5. Questionnaire | 26 |
| 6. Biological Sample Collection and Processing | 26 |
| 7. Complete Blood Cell Count (CBC) | 27 |
| 8. Circulating Angiogenic Cell (CAC) Quantification | 27 |
| 9. Platelet Mononuclear Cell Aggregate (PMA) Quantification | 29 |
| 10. Urine Metabolite Quantification | 29 |
| a. Creatinine | 29 |
| b. Volatile Organic Compounds (VOCs) and Tobacco Alkaloids | 30 |
| 11. Statistical Analyses | 30 |

| | |
|---|----|
| B. Murine Studies | 32 |
| 1. Animals and Treatment..... | 32 |
| 2. Exposures | 33 |
| a. Mainstream Cigarette Smoke (MCS)..... | 33 |
| b. Electronic Cigarettes (E-cigs)..... | 33 |
| c. Propylene Glycol and Vegetable Glycerin (PG:VG)..... | 34 |
| d. Formaldehyde (FR)..... | 35 |
| e. Acetaldehyde (AA)..... | 36 |
| f. Acrolein..... | 36 |
| g. Crotonaldehyde (CR)..... | 36 |
| 3. Urine Collection..... | 36 |
| 4. Urine Metabolite Quantification | 37 |
| a. Formate and Acetate | 37 |
| b. Volatile Organic Compounds (VOCs) and Tobacco Alkaloids..... | 37 |
| 5. Tissue Collection and Storage | 38 |
| 6. Complete Blood Cell Count (CBC) | 39 |
| 7. Plasma Biomarker Quantification..... | 39 |
| 8. Circulating Angiogenic Cell (CAC) Quantification | 39 |
| 9. Leukocyte Subpopulation Quantification | 40 |
| 10. Statistical Analyses | 41 |
| | |
| III. ASSOCIATIONS BETWEEN CIRCULATING ANGIOGENIC CELLS AND USE OF TRADITIONAL AND EMERGING TOBACCO PRODUCTS..... | 42 |
| A. Introduction..... | 42 |

| | |
|--|-----|
| B. Experimental Procedures | 43 |
| C. Results..... | 47 |
| D. Discussion..... | 64 |
| IV. SYSTEMIC, HEMATOLOGICAL, AND ENDOTHELIUM-RELATED EFFECTS OF MAINSTREAM CIGARETTE SMOKE, E-CIGARETTE AEROSOL, AND PROPYLENE GLYCOL:VEGETABLE GLYCERIN IN MICE | 74 |
| A. Introduction..... | 74 |
| B. Experimental Procedures | 75 |
| C. Results..... | 76 |
| D. Discussion..... | 101 |
| V. SYSTEMIC, HEMATOLOGICAL, AND ENDOTHELIUM-RELATED EFFECTS OF FORMALDEHYDE AND ACETALDEHYDE IN MICE | 109 |
| A. Introduction..... | 109 |
| B. Experimental Procedures | 110 |
| C. Results..... | 111 |
| D. Discussion..... | 133 |
| VI. SYSTEMIC, HEMATOLOGICAL, AND ENDOTHELIUM-RELATED EFFECTS OF ACROLEIN AND CROTONALDEHYDE IN MICE | 143 |
| A. Introduction..... | 143 |
| B. Experimental Procedures | 144 |
| C. Results..... | 145 |
| D. Discussion..... | 166 |

| | |
|--|-----|
| VII. ROLES OF EXTRACELLULAR SUPEROXIDE DISMUTASE AND TRANSIENT RECEPTOR POTENTIAL ANKYRIN 1 IN THE CARDIOVASCULAR EFFECTS OF TOBACCO PRODUCTS AND CONSTITUENT ALDEHYDES | 172 |
| A. Introduction..... | 172 |
| B. Experimental Procedures | 173 |
| C. Results..... | 174 |
| D. Discussion..... | 192 |
| VIII. SUMMARY AND CONCLUSIONS | 202 |
| A. Major Findings..... | 202 |
| B. Strengths | 211 |
| C. Limitations | 212 |
| D. Future Directions | 215 |
| E. Conclusions..... | 216 |
| REFERENCES | 219 |
| ABBREVIATIONS | 265 |
| CURRICULUM VITAE..... | 269 |

LIST OF TABLES

| TABLE | PAGE |
|--|------|
| Table 3.1. Antigenic identity of circulating angiogenic cells (CACs)..... | 46 |
| Table 3.2. Demographic characteristics by tobacco product use categories..... | 48 |
| Table 3.3. Medical history and medication use by tobacco product use categories | 51 |
| Table 3.4. Hematological measures by tobacco product use categories..... | 53 |
| Table 3.5. Mean urinary volatile organic compound (VOC) metabolite levels by tobacco product use categories (ng/mg)..... | 55 |
| Table 3.6. Percent change in levels of circulating angiogenic cells (CACs) comparing tobacco product use categories with never smokers in the fully adjusted model | 58 |
| Table 3.7. Percent change in levels of circulating angiogenic cells (CACs) comparing tobacco product use categories with never smokers in the secondary adjusted model | 61 |
| Table 4.1. Systemic parameters of male C57BL/6 mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 12 KY Reference 3R4F cigarettes)..... | 79 |
| Table 4.2. Hematological measures of male C57BL/6 mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 12 KY Reference 3R4F cigarettes)..... | 81 |
| Table 4.3. Systemic parameters of female C57BL/6 mice acutely exposed to either air or electronic cigarette aerosol (e-cig; JUUL Mango)..... | 88 |

| | |
|--|-----|
| Table 4.4. Hematological measures of female C57BL/6 mice acutely exposed to either air or electronic cigarette aerosol (e-cig; JUUL Mango) | 89 |
| Table 4.5. Systemic parameters of male C57BL/6 mice acutely exposed to either air or electronic cigarette aerosol (e-cig; blu Plus+) | 91 |
| Table 4.6. Hematological measures of male C57BL/6 mice acutely exposed to either air or electronic cigarette aerosol (e-cig; blu Plus+)..... | 92 |
| Table 4.7. Systemic parameters of female C57BL/6 mice acutely exposed to either air or propylene glycol:vegetable glycerin (PG:VG; 30%:70%) | 98 |
| Table 4.8. Hematological measures of female C57BL/6 mice acutely exposed to either air or propylene glycol:vegetable glycerin (PG:VG; 30%:70%) | 99 |
| Table 5.1. Systemic parameters of male C57BL/6 mice acutely exposed to either air or formaldehyde (FR; 1, 2, or 5 ppm) | 115 |
| Table 5.2. Hematological measures of male C57BL/6 mice acutely exposed to either air or formaldehyde (FR; 1, 2, or 5 ppm)..... | 116 |
| Table 5.3. Systemic parameters of female C57BL/6 mice acutely exposed to either air or formaldehyde (FR; 2 or 5 ppm) | 121 |
| Table 5.4. Hematological measures of female C57BL/6 mice acutely exposed to either air or formaldehyde (FR; 2 or 5 ppm)..... | 122 |
| Table 5.5. Systemic parameters of male C57BL/6 mice acutely exposed to either air or acetaldehyde (AA; 5 ppm) | 130 |
| Table 5.6. Hematological measures of male C57BL/6 mice acutely exposed to either air or acetaldehyde (AA; 5 ppm)..... | 131 |

| | |
|--|-----|
| Table 5.7. Systemic changes in female C57BL/6 mice acutely exposed to either air or acetaldehyde (AA; 5 ppm) | 134 |
| Table 6.1. Systemic parameters of male C57BL/6 mice acutely exposed to either air or acrolein (1 ppm) | 149 |
| Table 6.2. Hematological measurements of male C57BL/6 mice acutely exposed to either air or acrolein (1 ppm) | 150 |
| Table 6.3. Systemic parameters in male C57BL/6J mice chronically exposed to either air or crotonaldehyde (CR; 1 ppm) | 154 |
| Table 6.4. Hematological measures of male C57BL/6J mice chronically exposed to either air or crotonaldehyde (CR; 1 ppm) | 156 |
| Table 6.5. Systemic measures in male C57BL/6J mice acutely exposed to either air or crotonaldehyde (CR; 1 or 3 ppm) | 160 |
| Table 6.6. Plasma biomarkers in male C57BL/6J mice acutely exposed to either air or crotonaldehyde (CR; 1 or 3 ppm) | 161 |
| Table 6.7. Systemic measures in female C57BL/6J mice acutely exposed to either air or crotonaldehyde (CR; 1 ppm)..... | 162 |
| Table 7.1. Systemic parameters of male TRPA1-null mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 12 KY Reference 3R4F cigarettes) | 176 |
| Table 7.2. Hematological measures of male TRPA1-null mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 12 KY Reference 3R4F cigarettes) | 178 |

| | |
|--|-----|
| Table 7.3. Systemic measures in male TRPA1-null mice acutely exposed to either air or crotonaldehyde (CR; 1 ppm)..... | 179 |
| Table 7.4. Plasma biomarkers in male TRPA1-null mice acutely exposed to either air or crotonaldehyde (CR; 1 ppm)..... | 181 |
| Table 7.5. Systemic changes in male WT or ecSOD-Tg mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 6 KY Reference 3R4F cigarettes)..... | 188 |
| Table 7.6. Systemic changes in male WT or ecSOD-Tg mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 12 KY Reference 3R4F cigarettes)..... | 190 |
| Table 7.7 Plasma biomarkers in male WT or ecSOD-Tg mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 6 KY Reference 3R4F cigarettes)..... | 193 |
| Table 7.8. Plasma biomarkers in male WT or ecSOD-Tg mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 12 KY Reference 3R4F cigarettes)..... | 194 |

LIST OF FIGURES

| FIGURE | PAGE |
|--|------|
| Figure 3.1. Flow cytometry gating scheme for circulating angiogenic cell (CAC) populations | 45 |
| Figure 3.2. Relative feature importance of demographic variables on levels of circulating angiogenic cells (CACs) | 60 |
| Figure 3.3. Interrelationships between circulating angiogenic cells (CACs) associated with e-cig or dual use compared with never smokers | 63 |
| Figure 4.1. Urinary metabolites after acute mainstream cigarette smoke (MCS) exposure | 78 |
| Figure 4.2. Effects of acute mainstream cigarette smoke (MCS) exposure on circulating stem cells in male mice | 82 |
| Figure 4.3. Urinary metabolites after acute electronic cigarette aerosol (e-cig; JUUL VA Tobacco) exposure | 84 |
| Figure 4.4. Urinary metabolites after acute electronic cigarette aerosol (e-cig; JUUL Mango) exposure | 85 |
| Figure 4.5. Urinary metabolites after acute electronic cigarette aerosol (e-cig; JUUL Menthol) exposure | 86 |
| Figure 4.6. Effects of acute electronic cigarette (e-cig; JUUL Mango) aerosol exposure on circulating stem cells in female mice | 90 |

| | |
|--|-----|
| Figure 4.7. Effects of acute electronic cigarette (e-cig; blu Plus+) aerosol exposure on circulating stem cells in male mice | 94 |
| Figure 4.8. Urinary metabolites after acute propylene glycol:vegetable glycerin (PG:VG; 30%:70%) exposure | 95 |
| Figure 4.9. Urinary metabolites after acute propylene glycol:vegetable glycerin (PG:VG; 50%:50%) exposure | 96 |
| Figure 4.10. Effects of acute propylene glycol:vegetable glycerin (PG:VG; 30%:70%) exposure on circulating stem cells in female mice | 100 |
| Figure 5.1. Urinary formate levels in formaldehyde- or tobacco product-exposed male mice | 113 |
| Figure 5.2. Effects of two-week formaldehyde (FR; 5 ppm) exposure on circulating stem cells in male mice..... | 118 |
| Figure 5.3. Effects of acute formaldehyde (FR; 5 ppm) exposure on circulating stem cells in male mice | 123 |
| Figure 5.4. Effects of acute formaldehyde (FR; 2 ppm) exposure on circulating stem cells in male mice | 124 |
| Figure 5.5. Effects of acute formaldehyde (FR; 2 ppm) exposure on circulating stem cells in female mice..... | 125 |
| Figure 5.6. Effects of acute formaldehyde (FR; 5 ppm) exposure on circulating stem cells in female mice | 126 |
| Figure 5.7. Urinary acetate levels in acetaldehyde- or tobacco product-exposed male mice..... | 128 |

| | |
|---|-----|
| Figure 5.8. Effects of two-week acetaldehyde (AA; 5 ppm) exposure on circulating stem cells in male mice..... | 132 |
| Figure 5.9. Effects of acute acetaldehyde (AA; 5 ppm) exposure on circulating stem cells in male mice..... | 135 |
| Figure 5.10. Effects of acute acetaldehyde (AA; 5 ppm) exposure on circulating stem cells in female mice | 136 |
| Figure 6.1. Urinary 3HPMA levels in acrolein- or tobacco product-exposed male mice | 147 |
| Figure 6.2. Effects of acute acrolein (1 ppm) exposure on circulating stem cells in male mice..... | 151 |
| Figure 6.3. Urinary HPMMA levels in crotonaldehyde- or tobacco product-exposed male mice..... | 153 |
| Figure 6.4. Effects of chronic crotonaldehyde (CR; 1 ppm) exposure on circulating stem cells in male mice..... | 158 |
| Figure 6.5. Effects of acute crotonaldehyde (CR; 1 ppm) exposure on circulating stem cells in male mice..... | 164 |
| Figure 6.6. Effects of acute crotonaldehyde (CR; 3 ppm) exposure on circulating stem cells in male mice..... | 165 |
| Figure 6.7. Effects of acute crotonaldehyde (CR; 1 ppm) exposure on circulating stem cells in female mice | 167 |
| Figure 7.1. Effects of acute crotonaldehyde (CR; 1 ppm) exposure on circulating stem cells in male TRPA1-null mice..... | 183 |

| | |
|---|-----|
| Figure 7.2. Urinary 3HPMA & HPMMA levels after acute mainstream cigarette smoke (MCS) exposure | 184 |
| Figure 7.3. Total urinary nicotine metabolite levels after acute mainstream cigarette smoke (MCS) exposure..... | 187 |
| Figure 7.4. Effects of acute mainstream cigarette smoke (MCS; 6 or 12 cigarettes) exposure on circulating stem cells in male WT and ecSOD-Tg mice | 195 |

CHAPTER I

INTRODUCTION

A. Background and Rational

1. Cardiovascular Disease and Air Pollution

Cardiovascular disease (CVD) is the leading cause of death both nationally (1) and globally (2). In 2016, the World Health Organization reported that approximately 27% of the 56.9 million deaths worldwide were due to ischemic heart disease and stroke; these diseases have been the leading causes of death globally for the last 20 years (2). In the United States, the American Heart Association reports that approximately 1 of every 3 deaths is due to CVD, with about 2,300 daily deaths related to some form of CVD (1). On a local level, between 2014-2016, Kentucky had a CVD mortality rate of 258.5 per 100,000 people, exceeding the national CVD mortality rate of 220.7 per 100,000 people by approximately 17% and ranking the state as the fifth highest in the nation for CVD mortality (1). The American Heart Association projects that CVD prevalence will continue to increase, with approximately 45.1% of the United States population expected to have some form of CVD by 2035 (1). In terms of cost, the average annual direct and indirect cost of CVD-related care in the United States in 2014 to 2015 was \$351,200,000,000, an amount which accounted for 14% of the total health expenditures for the county in that

year (1). As CVD prevalence increases, the cost of CVD-related health care is also expected to increase, with estimates of total direct CVD-related costs of approximately \$749,000,000,000 per year by 2035 (1).

Although there are many risk factors for the development of CVD (3), a better understanding of the relationship between environmental factors and health outcomes has led to the hypothesis that environmental exposures to air pollutants contribute to CVD development. Over the past 30 years, a number of clinical and epidemiological studies have established the damaging effects of air pollution on human health. The majority of this damage is found in the pulmonary and cardiovascular systems, leading to an increased risk of heart disease or stroke (4-13). Many of these health effects are linked to different forms of air pollution, including ambient air pollution and diesel and gasoline engine exhausts. Ambient air pollution is composed of a gaseous suspension of aerosolized particles, known as particulate matter (PM), that typically have an aerodynamic diameter <10 μm (14, 15); this range is classified into specific particle sizes: ultrafine (<0.1 μm), fine ($\text{PM}_{2.5}$, 0.1-2.5 μm), and coarse (PM_{10} , >2.5-10 μm). General exposure to ambient air pollution has been shown to be a significant trigger for heart attacks (4), and there is strong evidence linking increased levels of $\text{PM}_{2.5}$ with increased cardiovascular effects (15-17). A 10 $\mu\text{g}/\text{m}^3$ increase in $\text{PM}_{2.5}$ levels is associated with an increased risk of heart failure hospitalization (18) and with long-term all-cause mortality (5-9).

Additional studies of specific types of air pollutants further strengthen the link between exposure and cardiovascular events. $\text{PM}_{2.5}$ exposure significantly increases left ventricular mass (10) and systolic (11, 19, 20) and mean ambulatory arterial (21) blood pressures. Changes in $\text{PM}_{2.5}$ are associated with the onset of heart attacks (22) and

increased intima media thickness progression (12, 13), with accelerated carotid intima media thickness (CIMT) progression of up to 5.5 μm (per 10 $\mu\text{g}/\text{m}^3$) per year (23). Similarly, exposure to diesel exhaust and traffic-related pollution increases arterial stiffness (24) and systolic blood pressure (25) and is associated with both increased mortality hazard ratio and increased risk of dying after hospital discharge (26). Collectively, these pathogenic changes (e.g., increased CIMT, increased blood pressure) suggest that an underlying vascular alteration or dysfunction is responsible, and thus, there is a need to explore mechanisms consistent with these vascular manifestations.

Brook et al. (16) propose three likely mechanisms by which air pollutants may contribute to the development of CVD: 1) an imbalance in the autonomic nervous system; 2) translocation of particles, specifically ultrafine particulate matter, into the circulation; and, 3) the development of systemic oxidative stress and inflammation within the vasculature. These hypothetical pathways are generally accepted (17, 21, 24, 27-32). A primary outcome of each of these mechanisms is the initiation of endothelium dysfunction, which appears central (perhaps as a result of or in combination with oxidative stress and inflammation) to further development of CVD. Evidence suggests a strong association between endothelium dysfunction and cardiovascular events, leading to high morbidity and mortality from CVD (33) as well as other disease states (34-39). Nonetheless, more research is required in order to ultimately improve pollutant-related health outcomes. Based on the health and financial burden of CVD, it is necessary from medical, legislative, and economic standpoints to continue working towards decreased air pollutant exposures in an effort to collectively reduce morbidity and mortality and to reveal the biological mechanism(s) by which air pollutants cause and/or exacerbate CVD.

2. Reactive Oxygen Species

Oxidative stress as proposed by Brook et al. (16) is the result of excessive production of reactive oxygen species (ROS) (40-42). ROS are oxygen-containing, highly reactive molecules, many of which are endogenously produced by normal cellular metabolism (42, 43). At normal levels, ROS have been shown to have a number of beneficial aspects, serving as signaling molecules (43, 44) and playing roles in gene expression, cell growth, and apoptosis (42, 43). However, stressors such as pollutants or toxicants can lead to increased production of ROS (32, 45, 46), overcoming the body's ability to clear these oxidizing compounds and leading to the development of oxidative stress (40-42, 44, 47). Increased ROS and the resulting oxidative stress have been linked to a number of negative health outcomes (42, 47, 48), including CVD (42, 48-52).

Increased ROS and oxidative stress have been linked to downstream health outcomes via changes in the production and bioavailability of nitric oxide (NO), a potent vasodilator (40-42, 49). Excess levels of the anion superoxide in particular have been directly implicated in disease development via the development of endothelium dysfunction (40-42). These excess levels can occur when superoxide dismutase (SOD), the antioxidant enzyme that catalyzes the dismutation of superoxide, is inactivated or mutated (53). The free superoxide molecule can then bind with NO to form peroxynitrite, a powerful oxidant that can subsequently cause dysfunction of endothelial nitric oxide synthase (eNOS), one of the enzymes responsible for synthesizing NO (40, 41, 49, 50). By disrupting eNOS function, NO levels are further decreased, leading to an imbalance in the vasoactive factors in the vasculature and resulting in endothelium dysfunction (40, 41). This dysfunction has been noted as one of the earliest vascular changes in a number of

disease states, including atherosclerosis (54-56) and both systemic (57) and pulmonary (58) hypertension. Although a number of therapeutic strategies specifically targeting the production of ROS and the development of endothelium dysfunction have been developed for the treatment of CVD (59-61), the relationship between these endothelial and vascular changes and downstream disease development is complex, and more research is needed to fully understand these connections.

3. Circulating Angiogenic Cells

Once endothelium dysfunction develops, repair is at least partially mediated by circulating angiogenic cells (CACs) (62, 63). CACs are sensitive indicators of both vascular health and pollution exposure, and they play an important role in proper endothelial function and overall endothelial health (63-66). These cells are recruited from the bone marrow to sites of endothelial damage where they contribute to repair of the vasculature (62, 67-69). Vasa et al. (70) found that the number of atherosclerotic risk factors, such as hypertension, diabetes, and hypercholesterolemia, was inversely correlated with CAC levels and CAC function. Similarly, Hill et al. (66) demonstrated a strong correlation between an individual's Framingham Risk Score, the 10-year risk score for nonfatal heart attack or sudden cardiac death, and their CAC levels, with higher risk scores associated with diminished CAC levels. Furthermore, the group showed a significant relationship between endothelial function as measured by flow-mediated brachial reactivity and CACs (66).

Based on these results, Hill et al. concluded that CACs were a better predictor of vascular reactivity than the presence or absence of conventional risk factors and that CACs may serve as a surrogate biologic marker for vascular function and cumulative

cardiovascular risk (66). This concept has been evaluated in a number of studies examining the relationship between CVD and air pollution. Consistent with Hill's theory, these studies show that exposure to air pollution causes a decrease in CAC levels that correlates with an increased CVD risk (67, 71-74). Therefore, it is imperative for any research concerning CVD and air pollution to assess CAC levels and function in relation to endothelium dysfunction and as an indicator of vascular homeostasis.

4. Tobacco Smoke and Cardiovascular Disease

It is also important to consider personal sources of pollutant exposures when conducting CVD research. While some level of exposure to ambient air pollution is inevitable (4), exposure to indoor air pollutants can be controlled to a degree; one indoor air pollutant that is of particular health concern is tobacco smoke. Tobacco smoke is the most significant modifiable risk factor in the development of CVD (1, 14, 75-77). Cigarettes contains numerous toxic compounds, many of which could contribute to disease development (30, 78-82). The Centers for Disease Control and Prevention (CDC) reports that although cigarette use has been declining for a number of years, reaching an all-time low of 13.7% in 2018 (83), tobacco use still remains the leading cause of preventable death both globally and nationally, with overall mortality for American smokers three times that of their fellow non-smokers (1). Furthermore, secondhand smoke (SHS) has also been shown to cause a large number of negative health effects. The CDC reports that since 1964, approximately two and a half million nonsmokers have died from health problems caused by exposure to SHS (84). The Surgeon General's report estimates more than 33,000 nonsmokers die every year in the United States from coronary heart disease caused by exposure to SHS (80). It has also been shown that SHS exposure can cause heart attacks

and strokes in nonsmokers (80, 84), and a variety of health consequences in both children and adults have been linked to SHS exposure as well, including cardiovascular, respiratory, and reproductive effects (80, 84). Much as with ambient air pollutant exposure, the development of oxidative stress and endothelium dysfunction likely contributes to this disease development, as both mainstream cigarette smoke (MCS) (85, 86) and SHS (87, 88) exposures have been linked to increased production of ROS.

5. Aldehydes

Aldehydes, one class of compounds known as volatile organic compounds (VOCs), are compounds with a terminal carbonyl group (-CHO), and they can be classified as saturated, unsaturated, or aromatic. Unsaturated aldehydes contain one or more carbon-carbon double bond, which increase their reactivity compared with saturated aldehydes (89). These compounds are generally ubiquitous in the natural environment, and many are found in foods and drinks, including meats, cheeses, fried foods, bread, and beer and other alcohols (90). However, the majority of environmental aldehydes are generated from anthropogenic sources, including combustion sources (e.g., diesel and gasoline exhaust, industrial emissions, MCS, and SHS) and also various consumer products and building materials that leach aldehydes (90). Additionally, as they have been linked to a number of negative health effects (91-94), aldehydes from both ambient and personal exposure sources (e.g., MCS, SHS, occupational exposures, personal hobby exposures) present potential health risks. Because of their pervasiveness and their documented toxicity, four specific aldehydes have been identified as significant contributors to disease risk and development: formaldehyde, acetaldehyde, acrolein, and crotonaldehyde.

a. Formaldehyde and Acetaldehyde

The cigarette- and pollutant-related development of CVD may in part be attributable to saturated aldehydes such as formaldehyde (FR) and acetaldehyde (AA). Both of these aldehydes have been officially established by the United States Food and Drug Administration as harmful constituents in tobacco products and tobacco smoke, with FR listed as a respiratory toxicant and a carcinogen and AA listed as a carcinogen, a respiratory toxicant, and an addictive component (82, 93, 95). Furthermore, the Institute of Medicine ranked AA and FR as two of the most significant toxins in tobacco smoke in relation to CVD (96). There is some level of natural exposure to both of these products, both exogenously and endogenously. FR, the smallest aldehyde, is naturally produced in the cells of humans and other mammals by amino acids and via the demethylation of various endogenous metabolites (93, 97, 98). It is swiftly degraded via FR dehydrogenase and S-formylglutathione hydrolase to form formic acid (93, 97, 99) and does not therefore accumulate in the blood, with a half-life of approximately 1.5 minutes (99, 100), contributing to a basal concentration of 2-3 mg/L (93, 97, 99). In terms of exogenous sources, FR is released as a combustion product, both of biomass (101) and fuel (102, 103). Combustion accounts for the majority of FR released in the environment (102). FR is also used in many consumer products, including carpets, manufactured wood products, paints and varnishes, and preservatives, which may lead to increased exposure (102). However, levels of FR in the air are typically low, (100, 102, 104), with natural concentrations less than 1 $\mu\text{g}/\text{m}^3$ (93, 99), although some variation is seen between rural, suburban, and urban locations (93, 99, 102).

AA, the second smallest aldehyde, is pervasive in the ambient environment and is

widely used in different industries as an intermediate in the production of other chemicals (105, 106). It is produced due to the incomplete combustion of wood from both personal and industrial use (105, 106), and it is also naturally found in dairy, meats, and plants commonly consumed by humans (105). Alcohol consumption is another common source of exposure (105, 106), as AA is formed from the breakdown of ethanol via alcohol dehydrogenases. The concentration in ambient air is typically between 5 and 10 $\mu\text{g}/\text{m}^3$ (92, 107). Like FR, AA has a short half-life in the blood—again, approximately 90 seconds (108, 109)—and does not typically accumulate in the blood; Lynch et al. report the concentration of AA in the whole blood of fasted subjects to be less than 0.1 mg/L (110).

Although some exposure to both FR and AA is common for all individuals, those exposed to MCS or SHS are exposed to much higher levels of these compounds, greatly increasing their risk of morbidity and mortality (92, 93, 95, 99, 111-113). The World Health Organization reports that, on average, a person is naturally exposed to approximately 1 mg of FR per day; smoking 20 cigarettes per day will increase that exposure, raising levels of inhaled FR by an additional 0.9-2.0 mg/day (99). Direct analysis of MCS has measured levels of FR between 12-29 $\mu\text{g}/\text{cigarette}$ (114-117), and additional exposure from SHS can increase FR exposure by 0.2-3.5 mg/day taking into account exposure possible in both the workplace and at home (99).

Typical exposure to AA, although ubiquitous, occurs via food intake, particularly alcohol consumption (105-107). Therefore, any significant level of exposure via inhalation can significantly increase overall exposure, and tobacco smoke is one of the most common modifiable forms of exposure to AA (107, 118). Based on the mean ambient concentrations of AA in both indoor and outdoor environments, Bruinen de Bruin et al.

(107) estimate that an individual is exposed to 0.321 mg of AA a day. The current literature reports a range of AA within cigarettes, but most data suggest between 0.5-3 mg/cigarette (107, 109, 114, 117, 118). Based on 2016 data showing that American daily smokers consume an average of 14 cigarettes per day—a level that is down from the estimated 17 cigarettes per day in 2005—this increases the average smoker’s exposure to AA by 7-42 mg/day (119). Exposure to AA via SHS may be equivalent or even greater than that from MCS (107, 118), with reported levels ranging from 2-3 mg/cigarette (107).

Some cardiovascular and hematopoietic effects of FR or AA exposure are documented, but there remain questions regarding the mechanisms through which these aldehydes cause cardiovascular effects. Exposure to FR has been shown to cause hematotoxicity, significantly decreasing total leukocyte count (93, 120-122) as well as platelets and red blood cell counts (93, 120, 122). Progenitor cell lines were additionally seen to be decreased with exposure (120, 122). FR has also been shown to cause decreases in blood pressure; in rats, inhalation of formalin, a saturated solution of FR in water, produced a significant drop in arterial blood pressure (123), and *in vitro* studies also note a concentration-dependent relaxation in response to FR (124-127). Hypotension has also been noted in individuals who have attempted suicide via ingestion of FR (128). Furthermore, occupational exposures in industries associated with increased exposure to FR (embalmers, undertakers, perfumery workers) have been associated with increased incidence of heart disease (129-131).

Human studies of the cardiovascular effects of AA typically revolve around aldehyde dehydrogenase (ALDH2), the fast-acting enzyme that metabolizes AA, and specifically the mutation in the enzyme that prevents AA metabolism and allows for

increased blood levels of AA (132). This mutation is present in 35-45% of Asians and causes facial flushing and nausea after alcohol consumption, presumably due to high levels of AA in the blood (133). The resulting increase in AA has been linked to several negative cardiovascular outcomes, including increased risk of hypertension (134, 135), coronary heart disease (136, 137), and heart attack (136), as well as alterations in heart rhythm (132, 138) and overall cardiac toxicity (132, 135). Other studies have observed that prolonged exposure to AA can cause decreases in both leukocytes and red blood cells (139), and the formation of hemoglobin-AA adducts decreases the amount of free hemoglobin available in the blood, which could contribute to anemia (139-142).

b. Acrolein

Unsaturated aldehydes, aldehydes that contain at least one carbon-carbon double bond, have also been linked to the development of CVD (71, 74, 86, 143-145). Unsaturated aldehydes are more reactive than their saturated counterparts as a result of the polarization of their carbon-carbon double bond by the aldehyde group, which increases the potential for nucleophilic addition (146). One unsaturated aldehyde that has been extensively researched is acrolein. Acrolein is a combustion air pollutant associated with tobacco smoke, vehicular exhaust, open fires, and industrial emissions (94). Although acrolein can be found in the environment, levels are typically low, and the highly reactive nature of the compound gives acrolein a half-life of roughly one day (94). Exposure more commonly occurs via ingestion of foods such as beer, cheese, bread, coffee, and fried or burnt foods, and some level of endogenous production of acrolein occurs via lipid peroxidation (94). Although both ambient and indoor levels of acrolein are typically low, ranging between <1 and 12 ppb, indoor levels can become significantly increased by tobacco smoke. MCS is

known to be a significant source of acrolein, with between 18-98 $\mu\text{g}/\text{cigarette}$ or 50-70 ppm of acrolein generated per puff in MCS (90, 114, 117, 147); this level is significantly higher than the 4-8 ppm generated by exhaust from gasoline and diesel engines (148).

The Agency for Toxic Substances and Disease Registry classifies acrolein as an eye, nose, skin, and respiratory irritant, and the United States Department of Health and Human Services notes that acrolein may be a possible human carcinogen (94). Acrolein has been shown to also contribute to the development of CVD. Haussmann (145) reports that almost 90% of the non-cancer health risk associated with smoking, particularly the risk of cardiovascular and pulmonary diseases, is thought to be due to acrolein, and Stabbert et al. (149) have shown that the majority of the cytotoxicity related to the gas vapor phase constituents of MCS is due to acrolein. Furthermore, exposure to acrolein has been linked to increases in CVD risk in both humans and mice (71, 74, 150-153); inhalation of acrolein even at levels low enough to not induce a respiratory response showed changes in cardiac performance (154). Inhalation of acrolein has also been shown to cause endothelium dysfunction in mice (150) and to decrease levels of CACs in both humans (71) and murine models (74, 151), while ingestion of acrolein promotes pro-atherosclerotic changes (152, 153).

c. Crotonaldehyde

Crotonaldehyde (CR) is another unsaturated aldehyde found in high levels in MCS and linked to CVD. CR is found in many foodstuffs, including meat, fruits, vegetables, milk, and bread (155, 156), and there is some endogenous production of CR via lipid peroxidation and the metabolism of 1,3-butadiene (157). However, most exposure to CR occurs through anthropogenic sources such as industry emissions, engine exhaust, wood

smoke, and tobacco smoke (155-157). Because CR is a ubiquitous compound, low levels of exposure via ingestion or inhalation are expected on a day-to-day basis (158), but most exposure occurs via cigarette smoke (156, 159). The concentration of CR in MCS is estimated to be between 1-53 $\mu\text{g}/\text{cigarette}$, although this level may vary somewhat based on the type of cigarette (160). However, a recent study by Jaccard et al. (117) reported a CR yield of approximately 10 $\mu\text{g}/\text{cigarette}$ in 3R4F Kentucky reference cigarettes, which are commonly used as standard cigarettes for non-clinical tobacco research (161).

CR, like acrolein, is classified as an eye, nose, skin, and respiratory irritant, and inhalation of even low levels of CR has been reported to cause extreme respiratory irritation (157). The United States Environmental Protection Agency further classifies CR as a possible human carcinogen (162). Additionally, CR has been linked to the production of oxidative stress (163, 164), the dysfunction of cardiomyocyte contractility (164), and the development of atherosclerosis in African-American men occupationally exposed to 1,3-butadiene, the parent compound of CR (165, 166). Similarly, inhalation of 1,3-butadiene was shown to accelerate the development of arteriosclerotic lesions in cockerels (167). However, despite these findings and despite the physical and chemical similarities between CR and acrolein, more research is needed to further elucidate the role of CR in the development of CVD.

6. Electronic Cigarettes

A recent report by the CDC found that smoking rates among American adults were down to 13.7% in 2018, an all-time low set by the continuing decline in smoking rates over the past few decades (83). However, a new class of nicotine-delivery devices has recently been introduced, giving traditional tobacco users a new route for their nicotine

consumption. Electronic nicotine delivery systems (ENDS) deliver an inhalable aerosol created by heating a solution known as an e-liquid. Liquids in ENDS are composed of a humectant such as propylene glycol (PG) and/or vegetable glycerin (VG) and typically contain nicotine (168-171); many users also use flavor additives (96, 172-174). The most commonly used ENDS are electronic cigarettes (e-cigs) (173, 175-177). Although the first patent for a smokeless non-tobacco cigarette was first filed in the United States in 1965 (178), e-cigs did not become widely available until the early 2000s (173). E-cigs are simply designed, consisting of a battery, a cartridge to contain the e-liquid, and a heating element to vaporize the e-liquid and produce the aerosol (96, 173, 175, 179). Different generations of e-cigs vary somewhat in appearance, but these basic elements are all still present (175, 180).

Usage of ENDS devices has increased dramatically over the past several years, with a significant rise in the use of e-cigs. The 2018 National Health Interview Survey estimated that 49.1 million American adults used any tobacco product, with approximately of 3.2% (8.1 million) of individuals reporting use of a tobacco product other than traditional cigarettes reporting use of e-cigs (83). Among total tobacco product users, 42.6% reported daily use of e-cigs, and of the 9.3 million current dual-use tobacco product users, more than 30% report using both traditional cigarettes and e-cigs (83). In another national sampling of adults in the United States, 84.5% of e-cig users reported usage as a smoking cessation tool or a healthier alternative (181). Because the long-term health effects of e-cigs are still unknown, the increased use of e-cigs among adults may beget worrisome health conditions in the future, but more alarming is the trend in e-cig use among youth. E-cigs have been the most commonly used tobacco product among American middle (grades 6-8) and high

school (grades 9-12) students since 2014 (182). The 2018 National Youth Tobacco Survey estimated that 20.8% of high school students and 4.9% of middle school students were current e-cig users, with current use defined as usage on one or more day(s) during the past month, with exclusive use of e-cigs reported by 42.0% and 42.7% of high school and middle school students e-cig users, respectively (182). Further analysis of these data showed that although there was no significant change in use of any tobacco product overall among high school and middle school students during 2011-2018, there were significant nonlinear increases in e-cig use specifically among both age groups (182); a 77.8% increase among high school students and a 48.5% increase among middle school students were reported in 2017-2018 alone despite no other significant changes in the use of other tobacco products occurring during this time period (182). The 2019 National Youth Tobacco Survey showed that usage has further increased, with an estimated 27.5% of high school students and 10.5% of middle school students reporting current use of e-cigs, with the majority of e-cig only users using flavored e-cigs (183).

As previously mentioned, e-cig use is commonly cited as a means of smoking cessation or as a healthier alternative to traditional tobacco smoking (173-175, 177, 181, 184). This latter claim is largely due to the fact that e-cigs do not contain actual tobacco leaf, nor does their use involve combustion (175, 180). However, the true health effects of these devices are currently unknown. Early studies of the health effects of e-cigs have shown increased reports of cracked teeth and tongue and/or inside-cheek pain in adolescent e-cig users (185), increased reports of dry mouth, sore throat, and acute cough in never-smokers and of acute cough in smokers with and without a history of respiratory disease (186), significantly increased airway resistance (187, 188), and impaired mesenchymal

stem cell differentiation (189). Studies in humans have also shown increases in markers of oxidative stress after vaping (190, 191), and levels of ROS have been measured in e-cig aerosol (192).

In mice, a two week exposure to e-cigs significantly increased levels of oxidative stress and impaired pulmonary anti-microbial defenses, leading to enhanced influenza A-induced illness and mortality (174). A similarly impaired pulmonary response to an infectious challenge was seen in mice exposed for four months to 60%:40% PG:VG alone (193). Other studies in mice link chronic e-cig exposure with aortic stiffness and impaired aortic endothelial function in female C57BL/6 mice (194) and to cardiac dysfunction, atherosclerotic development, and increased oxidative stress in apolipoprotein-E knockout mice (195). In rats, acute exposure to e-cig aerosol from either a JUUL device or from an earlier generation e-cig showed impaired endothelium function as measured by flow mediated dilation in both males and females (196). Furthermore, female rats exposed to e-cigs during pregnancy show decreased circulation in both the maternal uterine and fetal umbilical cords, indicating potential development of negative health effects in the next generation related to maternal e-cig use while pregnant, an outcome similar to that seen with the use of traditional cigarettes during pregnancy (197).

More studies have focused on the potential toxicity of the components within the aerosol produced by e-cigs. These analyses have revealed that the aerosol, like MCS, contains many harmful or potentially harmful components, including particulates (184, 198-202), metals (184, 203-205), and VOCs, including many aldehydes, (199, 201, 206-208), although possibly at lower levels than found in traditional cigarette smoke. Specific aldehydes, including FR, AA, and acrolein, have also been detected in e-cig aerosol (96,

180, 207-213). PG and VG, whether individually or together, have been shown to be responsible for some of this aldehyde generation (168, 214-216). Generated levels of unsaturated aldehydes, however, are much lower than those found in MCS and much lower compared with generated levels of saturated aldehydes, with CR showing little if any generation in different e-cig flavorings and humectants (96, 168).

However, the flavorings themselves could also be contributing to negative health outcomes, a concern on its own but even more so as most youths report using flavored e-cigs (183). In 2014, Zhu et al. (217) reported that there were at least 7,764 unique flavors of e-cig liquids sold by 466 distinct e-cig brands. This number has grown as the popularity of e-cigs has increased and as individuals have begun creating and using their own flavors. This wide variety of flavors may pose a health risk that is only beginning to be recognized. Many of the flavorings used to make flavored e-cig liquids come from food flavorings that are deemed by the Flavor and Extract Manufacturers Association as safe for ingestion but not necessarily for inhalation (218). Historically, the inhalation of aerosolized diacetyl, a flavoring that had been evaluated as safe for ingestion, led to the development of bronchiolitis obliterans in a number of microwave popcorn production plant workers (219), and many in the healthcare industry are fearful of the development of similar pulmonary diseases in response to the use of flavored e-cig liquids (220-223).

These fears seem to be founded, as, over the past year, a number of lung injury cases have been associated with e-cig use (224-230). The CDC reports that as of February 18, 2020, (the date of the last online update) more than 2,800 cases of e-cigarette, or vaping, product use associated lung injury (EVALI) have been reported from all 50 states, the District of Columbia, and 2 U.S. territories, and 68 EVALI deaths have been confirmed

(230). Of the admitted patients, approximately 76% are under the age of 35, with 15% aged under 18 years old (230), the age group most likely to be using e-cigs based on the appeal of the flavors rather than as a cessation aid (231, 232). Vitamin E acetate, a thickening agent used in many tetrahydrocannabinol (THC)-containing e-cig liquids, has been identified as the primary cause of EVALI (225, 230, 233), and many states have taken steps to prevent further e-cig-related illnesses, especially among youths, by placing temporary statewide bans on all or some flavored e-cigs and other ENDS (234-240).

In terms of cardiovascular outcomes, early studies of flavorings in endothelial cell lines have shown that flavors can pose a cardiovascular health threat through the development of endothelium dysfunction (241, 242), but more research is needed to better clarify the vascular toxicities that may be induced by e-cigs. There is also a need to determine how the presence of nicotine in these devices may contribute to adverse health effects. The cardiovascular effects of nicotine have been studied extensively in relation to traditional cigarettes, showing increased heart rate (243-245), blood pressure (243, 244, 246, 247), platelet aggregation (248, 249), and atherosclerotic development (250, 251). However, the amount of nicotine in e-liquids can vary substantially (252-255), which could have an impact on the use of e-cig devices as a harm reduction or cessation tool for traditional smokers and on new use from never-smoking individuals, particularly adolescents (256-259)

Regardless, it is clear that e-cigs are contributing to the development of disease in individuals with an otherwise low risk of disease, and although the specific cause of these illnesses has yet to be definitively identified, it is possible that the presence of saturated and unsaturated aldehydes, either alone or in combination with other constituents, in e-cig

aerosol may be contributing to the growing health threat posed by these devices.

7. Transient Receptor Potential Ankyrin 1 Channel

Unsaturated aldehydes such as acrolein have been shown to work through activation of the transient receptor potential ankyrin 1 (TRPA1) channel. The TRPA1 channel is a member of the TRP superfamily of cation channels and the only member of the ankyrin subfamily (260). TRPA1 is a non-selective cation (calcium) channel and promiscuous sensory receptor concentrated in sensory fibers that mediates pain reception/transmission as well as irritant-related pulmonary reflexes including cough, respiratory braking (slowing rate and depth of breathing), and bronchoconstriction (261). TRPA1 has been shown to be activated by a number of stimuli, including temperature (260, 262), pressure (262), and ROS (263-266), as well as by noxious compounds including diesel exhaust (267), cigarette smoke (261), acrolein (260, 262, 268, 269), cinnamaldehyde (260, 262, 266, 268, 270), and FR (127). The activation of the channel by the unsaturated aldehydes or compounds containing these aldehydes is proposed to occur through aldehyde conjugation with the TRPA1 N-terminal free cysteines (271).

Although TRPA1 was initially identified in sensory neurons (272), the channel has since been found in non-neuronal locations, including the endothelium (260, 263, 270, 272), urothelium (273, 274), vasculature (275, 276), and heart (277, 278). TRPA1 is known to primarily play a role in inflammatory and pain responses, likely through the release of vasoactive peptides such as substance P and calcitonin gene-related peptide that increase blood flow, vascular permeability, and leukocyte binding—a collective process referred to as ‘neurogenic inflammation’ (262, 272, 279-285). TRPA1 is also important in respiratory irritant responses (262, 279, 280, 286-288), and some studies have shown that

the channel helps mediate vasodilation through calcium influx (127, 265, 266, 270, 272, 275). Despite data showing that the negative cardiovascular effects of inhaled acrolein are TRPA1-dependent (261, 269, 289, 290), the role of TRPA1 in potential cardiovascular toxicity of other aldehydes and mixtures (e-cig aerosol) is not well known.

8. Statement of Goals

Early studies of e-cig aerosol exposure have shown the development of endothelium dysfunction similar to that seen with tobacco smoke. Many of these studies hypothesize that this dysfunction is related to shared constituents between MCS and e-cig aerosols, including acrolein, FR, and AA. Although previous research has linked acrolein with cardiovascular toxicity in both humans and mice, the effects of other aldehydes on endothelium function and repair are unknown. Therefore, the objective of this project is to evaluate the mechanism of tobacco product-induced CVD via the development of endothelium-related changes, as well as systemic and hematological effects.

B. AIMS AND PROPOSALS

1. Measure hematological and endothelium-related changes in humans in response to tobacco-product related pollutant exposures.

Both ambient and personal air pollutant exposures have been linked to decreased CAC levels in humans, but the health outcomes vary with different forms and durations of exposure. Thus, we will examine the response of CACs and other biomarkers of hematological change to personal exposures (tobacco use, e-cig use, dual product use) in a low to medium-risk heart disease human cohort. Measures of urinary metabolites will also be measured to determine whether VOCs found in MCS are generated in similar levels in

e-cigs and to establish the potential cardiovascular health risk of these compounds.

2. Measure systemic, hematological, and endothelium-related changes in mice in response to tobacco products and constituent aldehydes.

Exposures to both tobacco smoke and e-cig aerosol have been linked to the development of endothelium dysfunction and downstream CVD. Using C57BL/6J (wild type, WT) male mice, we will measure concentration- and duration-dependent changes in CACs and other hematological biomarkers as well as markers of systemic change following exposure to MCS, e-cig aerosol, and aldehydes (FR, AA, acrolein, and CR) found in tobacco smoke and/or e-cig aerosol as well as humectants (PG:VG) used in e-cig devices. We will also expose female WT mice to determine whether exposure-induced effects are sex-dependent.

3. Test the roles of ecSOD and TRPA1 in the development of hematological, systemic, and endothelium-related changes contributing to CVD.

The use of ecSOD-transgenic mice has been shown to reduce levels of ROS in response to ambient exposures, but these mice have not been extensively used to relate exposure to downstream vascular injury and disease. Likewise, mice lacking expression of the TRPA1 gene have been used to examine cardiopulmonary toxicity in response to direct exposure to unsaturated aldehydes, but these mice have not been extensively used to relate exposure to specific markers of downstream endothelium dysfunction and to changes in hematological and systemic biomarkers. We will thus use extracellular superoxide dismutase transgenic (ecSOD-Tg) mice—mice overexpressing extracellular superoxide dismutase in the lungs—and mice lacking expression of TRPA1 (TRPA1-null mice) to test the roles of ecSOD and TRPA1 in the mechanism of cardiovascular-related outcomes

resulting from *in vivo* aldehyde exposures.

C. Overall Aim of this Dissertation

This dissertation aims to address the significant and global problem of CVD. In particular, this research seeks to investigate the relationship between CVD and air pollutant exposures. The proposed studies will focus on the role of personal exposures to MCS and e-cig aerosols, as well as shared constituents, on systemic, hematological, and endothelium-related outcomes that have been linked to the development of CVD and will seek to identify the mechanism through which these compounds act. These studies will also work to determine whether these effects differ between male and females. The results of this research will contribute to the growing knowledge of CACs and their role in the development of CVD as well as to the field of environmental cardiology in relation to personal pollutant exposures. Furthermore, the results of this research have the potential to contribute to regulation regarding harmful and potentially harmful constituents of tobacco-based products and their contribution to cardiovascular toxicity.

The focus of this study is the relationships between CACs, systemic and hematological biomarkers, and exposure to pollutants, specifically tobacco-based aerosols and specific aldehydes found in MCS and/or e-cig aerosols. Chapter 3 explores the relationship between CACs and tobacco product-related exposures in a human cohort. Chapter 4 investigates the effects of MCS, e-cig aerosol, and PG:VG on CACs and biomarkers in murine models, while chapters 5 and 6 examine exposure-related changes from saturated and unsaturated aldehyde exposures, respectively. Chapter 7 examines the roles of ecSOD and the TRPA1 channel in the cardiovascular toxicity related to tobacco product use. Together, these data will provide novel insight into the cardiovascular impact

of traditional and emerging tobacco products and provide new understanding of the relationship between CACs and personal pollutant exposures.

CHAPTER II

EXPERIMENTAL PROCEDURES

A. Human Studies

1. Study Populations

To test the research hypothesis, a cohort of 143 participants with varying types of tobacco exposure was recruited for the Louisville Healthy Heart Study (LHHS) from September 2015 to April 2018. Participants were recruited through locally-placed advertisements, by word of mouth, or from nearby cardiovascular disease (CVD) clinics. Clinic enrollment was specific to adults undergoing primary or secondary preventative treatment for cardiovascular disease at University of Louisville and KentuckyOne Health hospitals clinic systems. Enrollment outside the clinic was focused on individuals currently using tobacco products, with a specific focus on individuals who were e-cigarette (e-cig) only users. All advertisements, study consent forms, and research protocols were approved through the University of Louisville's Institutional Review Board (IRB 09.0174).

2. Eligibility

Eligibility was determined based on age (≥ 18 years of age at the time of enrollment) and, when available, medical records in the general and preventative cardiology and family medicine clinics of the KentuckyOne Health hospitals located in and

around the University of Louisville's Health Science Campus in downtown Louisville, KY and from the Department of Public Health & Wellness screening database (set up previously under the direction of Drs. Troutman and Zahn by the Louisville Metro Health Department). Exclusion criteria included: 1) persons unwilling or unable to provide informed consent; 2) persons with significant and/or severe co-morbidities including the following conditions: significant chronic lung, liver, kidney, hematological, or neoplastic disease; chronic neurological or psychiatric illness; chronic infectious disease such as HIV or hepatitis; severe coagulopathies; drug/substance abuse; and chronic cachexia; 3) pregnant women; and 4) prisoners and other vulnerable populations.

3. Case Definition

For this study, primary prevention cases were defined as individuals who have known heart disease risk factors (e.g., hypertension, hypercholesterolemia, obesity, diabetes) but no evident coronary artery disease; secondary prevention individuals were those with evident heart disease in addition to heart disease risk factors that require treatment. Specifically, these latter individuals had a clinical manifestation of coronary artery disease and were being treated for risk factors. Fundamentally, the designation of a patient as a primary or secondary preventative case was based on the absence or presence of coronary artery disease.

4. Recruitment

Study participants had no restriction on geographic location of residence. Recruitment took place through local print and electronic advertisements, by word of mouth, and at nearby cardiovascular clinics. If recruitment occurred at a clinic, patients were approached during their clinic visit. Interested individuals were fully informed as to

the purpose of the study and their eligibility confirmed. Individuals willing to participate were either recruited immediately following their clinic visit or required to schedule a separate appointment at their convenience. At this recruitment appointment, individuals were asked to sign informed consent forms and administered a brief questionnaire to obtain demographic information, exposure history, and baseline clinical characteristics.

5. Questionnaire

All study participants were administered a questionnaire during their enrollment. This questionnaire was designed to collect relevant demographic and medical information and has been used in other human studies, both as originally written (71) and as modified (291). If previous medical history was available, the questionnaire was used to either confirm or to add to the clinically-indicated standard medical history, physical examination, and laboratory evaluation of traditional CVD risk factors preformed in the clinic. Additional questions sought information regarding the individual's demographic information, residential address, smoking history and current status, secondhand smoke (SHS) exposure, alcohol consumption, physical activity status, use of CVD medications, and CVD history including heart attack, heart failure, angina, high blood pressure, high cholesterol, diabetes, stroke, peripheral artery disease, aortic aneurism, and bleeding disorders. Affirmative answers for the presence of CVD risk factors were based on a professional diagnosis as reported by the patient. Answers were recorded in an electronic format, with the REDCap system (Research Electronic Data Capture; Vanderbilt University Medical Center, Nashville, TN) serving as an electronic database.

6. Biological Sample Collection and Processing

After informed consent was given, biological samples (blood and urine) were

obtained. A random catch urine sample was collected by the participant in the clinic or in the human studies laboratory when enrollment occurred outside the clinic. The urine samples were separated as follows: four 4.5 aliquots, four 2 mL aliquots, and two 4.5 mL aliquots with 45 μ L butylated hydroxytoluene as a preservation additive. Aliquots were stored at -80°C until the time of testing. A blood sample of approximately 25 mL was obtained via venipuncture by a licensed nurse or other medically-trained individual using standard procedures and precautions to ensure the safety of the participant and the nurse. If any participant became ill or uncomfortable, sample collection was discontinued until the participant recovered, and if the participant wished to withdraw from the study, they were allowed to do so at any time. Approximately 8 mL of blood was collected in a Vacutainer® CPT Mononuclear Cell Preparation Tube (Becton Dickinson; Franklin Lakes, NJ) for CAC analysis, 2.6 mL in a Vacutainer® ACD tube for platelet mononuclear cell aggregate analysis, and 6 mL total into a Vacutainer® Serum Tube, a Vacutainer® K₂EDTA Tube, and a Vacutainer® Citrate Tube for analysis of plasma components.

7. Complete Blood Cell Count (CBC)

A complete blood cell count (CBC) was performed on a Coulter Ac•T diff2 Hematology Analyzer (Beckman Coulter; Brea, CA). 18 μ L of collected blood from the Vacutainer® K₂EDTA tube were used for this analysis.

8. Circulating Angiogenic Cell (CAC) Quantification

Circulating angiogenic cell (CAC) populations in blood samples were characterized via fluorescence-activated cell sorting (FACS; FACSDiva v6.0 software; BD Biosciences; San Jose, CA) flow cytometry using established markers: CD34⁺ (endothelial progenitor), CD45^{+/-} (hematopoietic/non-hematopoietic), CD31⁺ (endothelial cells), and CD133⁺ (early

stem cell). Duda et al. (292) first described this method for defining populations of circulating endothelial and progenitor cells in human peripheral blood; a modified version of this protocol has been used in our laboratory to measure populations of human CACs (71, 73). Within 30 minutes of the blood draw, blood cells were separated in the CPT mononuclear separator tube by centrifugation at 1,600xg for 30 minutes at room temperature and then stored in an upright position at room temperature overnight. The following day, the upper layers of the sample were decanted into phosphate-buffered saline (PBS) and centrifuged at 500xg for five minutes at room temperature. The pelleted cells were then washed twice with 1% BSA/PBS buffer and then incubated with human FcR Blocking Reagent (Miltenyi Biotec) and a panel of fluorescently-conjugated antibodies on ice for 30 minutes. Fluorescent antibodies used included PE-labeled anti-CD34 (BD Biosciences), APC-labeled anti-AC133 (Miltenyi Biotec; Bergisch Gladbach, Germany), PE-Cy5.5-labeled anti-CD14 (Abcam; Cambridge, United Kingdom), APC-AlexaFluor 750-labeled anti-CD45 (Invitrogen; Carlsbad, CA), PE-Cy7-labeled anti-CD16 (BD Biosciences), FITC-labeled anti-CD31 (BD Biosciences), anti-CD41a (BD Biosciences), anti-CD235a (BD Biosciences), Pacific Blue (Pacific Blue monoclonal antibody labeling kit; Invitrogen), and a marker for dead cells (LIVE/DEAD fixable dead cell stain; Invitrogen). The cells were then washed again with 1% bovine serum albumin (BSA)/PBS, centrifuged at 500xg for five minutes at room temperature, and resuspended in 1% BSA/PBS.

Immediately following resuspension, events were collected by FACS using an LSR II flow cytometer (BD Biosciences) for a period of two minutes. Positive and negative boundaries for gating were determined using unstained controls, and sequential gating was

performed based on antigenic marker groupings. CAC counts were normalized to sample volume for analysis (71, 73).

9. Platelet Mononuclear Cell Aggregate (PMA) Quantification

A portion of the blood sample was collected in an ACD tube. One mL of this sample was fixed with a mixture of PBS and 4% paraformaldehyde and incubated on ice for 30 minutes. Red blood cells were then lysed with the addition of 24 mL of water and the sample centrifuged at 400 xg for 10 minutes at room temperature. Following centrifugation, the pelleted cells were resuspended in Tyrode's buffer and stored at 4°C overnight. The following day, 800 μ L of the sample was centrifuged at 1,000 xg for 5 minutes at room temperature then incubated on ice with BSA/Tyrode's buffer and FcR Blocking Reagent (Miltenyi Biotec) for 10 minutes. A second 30-minute incubation with a panel of fluorescently-conjugated antibodies followed, and finally the cells were centrifuged, washed, and resuspended in BSA/Tyrode's buffer. FACS was used to count platelet mononuclear cell aggregates (PMAs) on an LSR II flow cytometer as events that are both FITC (CD41⁺) and PerCP-Cy5.5 (CD45⁺) positive, with the final count calculated as the percentage of double-positive cells per total events (71).

10. Urine Metabolite Quantification

Collected urine was used to quantify levels of creatinine and volatile organic compounds (VOCs) and tobacco alkaloid metabolites as described below.

a. Creatinine

Concentrations of creatinine were measured on a Cobas Mira Plus Clinical chemistry Autoanalyzer (Roche; Basel, Switzerland) with Infinity Creatinine Reagent

(ThermoFisher Scientific; Waltham, MA). Urine was diluted 1:10 with deionized water up to 200 μ L for testing.

b. Volatile Organic Compounds (VOCs) and Tobacco Alkaloids

Urinary levels of volatile organic compound (VOC) metabolites, including the urinary metabolites of acrolein (3-hydroxypropylmercapturic acid, 3-HPMA) and crotonaldehyde (CR) (3-hydroxy-1-methylpropylmercapturic acid, HPMMA), and tobacco alkaloids metabolites were measured by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) as previously described with slight modifications (293). Briefly, 50 μ L of sample urine was combined with 400 μ L ammonium acetate (15 mM, pH 6.8) and 50 μ L internal standard and applied to an ACQUITY UPLC core system (Acquity UPLC HSS T3 column (150 mm x 2.1 mm, 1.8 μ M)) and a Quattro Premier XE triple quadrupole MS coupled with an electrospray source (Waters Corporation; Milford, MA). Ammonia acetate and acetonitrile were used as mobile phase for chromatographic separation. The peak areas of analytes were quantified using at least 12 data points across the measurement peaks, and TargetLynx 4.1 (Waters Corporation) was used for peak integration, calibration, and quantification of analyze concentrations. All values were normalized to urinary creatinine (mg/dL).

11. Statistical Analyses

Descriptive statistics were computed to describe the LHHS cohort and are presented as mean \pm SE for continuous variables and as n (%) for categorical variables. Population demographics, CVD history, medication use, markers of systemic injury, markers of thrombosis, and urinary VOC and tobacco alkaloid metabolites were compared across the tobacco product use categories based on self-reported product use and confirmed by

urinary cotinine levels. The tobacco product use categories used were never smokers, former smokers, sole cigarette users (CIG only), and sole e-cig users or dual users (E-cig or Dual users). Demographic characteristics were compared using one-way analysis of variance (ANOVA) with the Bonferroni post-hoc test for continuous variables and using χ -squared analysis with Fisher's Exact Test for categorical variables. Two participants were excluded from all analyses for misclassification based on inconsistencies between self-report of tobacco use and urinary cotinine levels.

Differences in CBC results between tobacco product use categories were tested using one-way ANOVA with the Bonferroni post-hoc test. Differences in levels of urinary VOC and tobacco alkaloid metabolites between tobacco product use categories were tested on log-transformed urinary VOC data using one-way ANOVA with the Bonferroni post-hoc test.

Relationships in the levels of CACs between tobacco product use categories were assessed on log-transformed CAC measures using multivariable-adjusted linear regression models with never smokers as the reference group. These models utilized the normal probability distribution and the identity link function. Models were initially adjusted for variables found to be potential confounders from the demographic analysis; these variables were age, sex, CVD history, use of CVD medications, family history of CVD, and SHS exposure. Models were also initially adjusted for socioeconomic status (SES) using annual household income as a proxy variable as previously reported (294, 295). A random forest model was subsequently used to determine which of the variables used in the fully adjusted model were most likely to have an effect on CAC levels independent of tobacco product use categories. The resulting relative feature importance plot indicated age and SES as

potential confounders, and a second model examining the relationship between CACs and tobacco product use categories was adjusted only for these two variables.

Analyses of demographic, CBC, and urinary VOC and tobacco alkaloid metabolite data by tobacco product use categories was performed using IBM SPSS Statistics V26 (IBM; Armonk, NY). Multivariable-adjusted linear regression modeling and random forest modeling were performed using R V3.6.1 (The R Foundation for Statistical Computing; Vienna, Austria) and utilizing the following packages: tidyverse V1.3.0 (296), forcats V0.4.0 (297), and randomForest V4.6.14 (298). Statistical significance assumed at $p < 0.05$. Data are expressed as indicated.

B. Murine Studies

1. Animals and Treatment

Male and female C57BL/6J (wild type, WT) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Male extracellular superoxide dismutase transgenic (ecSOD-Tg) mice (on a WT background) mice and male transient receptor potential ankyrin 1 (TRPA1)-null mice (on a WT background) were obtained from breeding colonies at the University of Louisville (Louisville, KY) (269, 299). All mice were treated according to the *Guiding Principles for the Care and Use of Animals in Research and Teaching* as adopted by the American Physiological Society, and all protocols were approved by the University of Louisville Institutional Animal Care and Use Committee (IACUC 16624). Before and during the exposures, mice were housed in the University of Louisville vivarium under pathogen-free conditions, controlled temperatures, and a 12h:12h light:dark cycle. Mice were maintained on a standard chow diet (Rodent Diet 5010, 4.5% fat by weight, LabDiet; St. Louis, MO).

2. Exposures

a. Mainstream Cigarette Smoke (MCS)

Male WT, male ecSOD-Tg, and male TRPA1-null mice were exposed to high-efficiency particulate air- (HEPA) and charcoal-filtered air (control) or mainstream cigarette smoke (MCS) for 4 days (6h/day) and euthanized immediately after the last exposure. MCS was generated in a state-of-the-art, 4-chamber *inExpose* system with a software-controlled cigarette smoking robot (Flexiware Software, SCI-REQ; Montreal, Canada) housed in the University of Louisville Inhalation Facility with a separate HVAC system to maintain barrier, temperature and humidity conditions. To generate MCS, a single reference cigarette (3R4F; University of Kentucky, Center for Tobacco Reference Products) was burned at a time (9 puffs, 35 ml/puff, 2 s/puff; 9 min/cigarette, 1 cigarette every 30 minutes) for a total of 12 cigarettes burned during the exposure period. Humidified cigarettes (60% 2-way humidity packet) (Boveda, Inc.; Wayzata, MN) were reloaded between each 2h session. The exposure chamber (5 L) had continuous air flow (3 LPM), and a MicroDust Pro (Casella; Bedford, United Kingdom) continuously measured smoke total suspended particulate (TSP) upstream of chamber. Post-chamber thimble filter collection provided gravimetric TSP data. Mice did not have access to food or water during the exposure.

b. Electronic Cigarettes (E-cigs)

Male and female WT mice were exposed to either HEPA- and charcoal-filtered air (control) or e-cigarette (e-cig) aerosol for a single 6h period or 4 days (6h/day) and euthanized immediately after the last exposure. A custom e-cig holder (SCI-REQ; Montreal, Canada) connected to a fully programmable, software-controlled (Flexiware

Software, SCI-REQ) exposure system was used to produce the e-cig aerosol. Blu Plus+ e-cigs with Classic Tobacco flavoring (BLU Products; Doral, FL) were used for the blu Plus+ exposure, and the accompanying blu Plus+ batteries and cartridges were purchased locally. JUUL e-cig liquids with Virginia (VA) Tobacco, Mango, and Menthol flavorings (Juul Labs, Inc.; San Francisco, CA) with blu Plus+ batteries were used for JUUL exposures.

The blu Plus+ e-cigs exposure was performed under the following conditions: 1) battery charged overnight; 2) new cartridge each day; 3) Classic Tobacco flavor (16 mg nicotine); 4) vaping protocol of 4s puff, 50-60 mL puff, 2 puffs/min x 9 min = 18 puffs = 1 session; 5) each 6h exposure had 20 sessions (1 session = 9 min vaping followed by 9 min bias air flow); and, 6) blu Plus+ LED lights during each puff (or is rapidly replaced with charged battery).

The JUUL e-cig exposures were performed under the following conditions: 1) battery charged overnight; 2) e-liquids placed in Mystic bridge cartomizer (Mistic Electronic Cigarettes; Charlotte, NC); 3) new cartomizer used each day; 4) VA Tobacco (5% nicotine), Mango, or Menthol flavor; 5) vaping protocol of 4s puff, 91 mL puff, 2 puffs/min x 9 min = 18 puffs = 1 session; 6) each 6h exposure had 20 sessions (1 session = 9 min vaping followed by 9 min bias air flow); and, 7) bias air flow continued for at least 15 min post-exposure to clear chamber.

Real-time monitoring (computer data logging) of TSP was done with an inline (pre-chamber) Microdust Pro near infrared detector (Casella; Bedford, United Kingdom), and humidity equilibrated gravimetric filters (post-chamber) were used throughout the exposure. Mice did not have access to food or water during the exposure.

c. Propylene Glycol and Vegetable Glycerin (PG:VG)

Male and female WT mice were exposed to either HEPA- and charcoal-filtered air (control) or 50%/50% or 30%/70% propylene glycol:vegetable glycerin (PG:VG) for a single 6h period or 4 days (6h/day). Aerosol from the PG:VG mixtures were generated using a refillable, clear tank (approximately 5 mL) atomizer with a coil resistance of 1.8 Ohm (Mistic Electronic Cigarettes; Charlotte, NC) coupled with a rechargeable battery from a blu Plus+ e-cig (3.7 V). The atomizer tank was weighed before and after the exposure session to determine solution consumption. The vaping protocol of the aerosolized 50%/50% PG:VG consisted of a 4s puff, 50-60 ml puff, 2 puffs/min x 9 min = 18 puffs = 1 session; each 6h exposure period has 20 sessions (1 session = 9 min vaping followed by 9 min bias air flow). The vaping protocol of the aerosolized 30%/70% PG:VG consisted of 4s puff, 91 ml puff, 2 puffs/min x 9 min = 18 puffs = 1 session; each 6h exposure period has 20 sessions (1 session = 9 min vaping followed by 9 min bias air flow). Mice did not have access to food or water during the exposures.

d. Formaldehyde (FR)

Male and female WT mice were exposed to either HEPA- and charcoal-filtered air (control) or formaldehyde (FR) for 4 days (2 or 5 ppm, 6h/day) or 2 weeks (5 ppm; 6h/day, 7 days/week) using a custom exposure system and certified permeation tubes (Kin-Tek; LaMarque, TX) as previously described (151). FR vapor was diluted with HEPA-filtered room air, and the FR concentration was continuously monitored using an in-line photoionization detector (MultiRae, Rae Industries; Sunnyvale, CA) upstream of a polycarbonate cage (about 31 L). Air or FR was delivered through a fine mesh screen at 8-10 L per minute via a custom cyclone-type top that distributed gas within 10% of the mean concentration at six locations in the cage (Teague Enterprises; Woodland, CA). Mice

did not have access to food or water during the exposure.

e. Acetaldehyde (AA)

Male and female WT mice were exposed to either HEPA- and charcoal-filtered air (control) or acetaldehyde (AA) for 4 days (5 ppm, 6h/day) or 2 weeks (5 ppm; 6h/day, 7 days/week) and euthanized immediately following exposure on the final day. The experimental protocol for the exposure is the same as described for FR above with the substitution of AA vapor.

f. Acrolein

Male WT mice were exposed to either HEPA- and charcoal-filtered air (control) or acrolein for 4 days (1 ppm, 6h/day) and euthanized immediately following exposure on the final day. The experimental protocol for the exposure is the same as described for FR above with the substitution of acrolein vapor.

g. Crotonaldehyde (CR)

Male and female WT mice and male TRPA1-null mice were exposed to either HEPA- and charcoal-filtered air (control) or crotonaldehyde (CR) for 4 days (1 or 3 ppm, 6h/day) or 12 weeks (1 ppm; 6h/day, 5 days/week) and euthanized immediately following exposure on the final day. The experimental protocol for the exposure is the same as described for FR above with the substitution of CR vapor.

3. Urine Collection

For each type of exposure, urine was collected using rodent metabolic chambers (Nalgene, Lab Products Inc.; Seaford, DE). After exposure, mice were placed singly per metabolic cage (Harvard Apparatus; Cambridge, MA) with 3% glucose/0.125% saccharin solution drinking water but without food to collect urine (in graduated cylinders in 4°C

water-jacketed organ baths) in 1h increments up to 3h post-exposure followed by a 3-16h (overnight, O/N; mice given food) urine collection (151, 300). Collected urine samples were centrifuged (1800xg, 5 min; to pellet any feces or food particles) in cold immediately after collection before being decanted and stored at -80°C.

4. Urine Metabolite Quantification

Collected urine was used to quantify levels of volatile organic compound (VOC) metabolites and metabolites of saturated aldehydes as described below.

a. Formate and Acetate

Urinary levels of formate and acetate, the metabolites of formaldehyde (FR) and acetaldehyde (AA), respectively, were measured by gas chromatography-mass spectrometry (GC-MS) as adapted and modified from previous reports (301, 302). In short, 20 µL of collected urine was mixed with 20 µL sodium phosphate (0.5 M, pH 8.0) containing ¹³C-formate (2.3 mM) and ¹³C₁-acetate (0.23 mM) internal standards, and pentafluorobenzyl bromide (130 µL, 0.1 M). The mixture was vortexed for 1 minute and then incubated at 60°C for fifteen minutes, and the resulting reaction products were extracted using hexane (300 µL) before being transferred to glass tubes for GC-MS analysis. The peak areas of analytes were quantified using at least 12 data points across the measurement peaks, and TargetLynx 4.1 (Waters Corporation) was used for peak integration, calibration, and quantification of analyze concentrations. Measured formate and acetate sample concentrations were corrected for the natural abundance of ¹³C isotopes.

b. Volatile Organic Compounds (VOCs) and Tobacco Alkaloids

Urinary levels of volatile organic compound (VOC) metabolites, including the

urinary metabolites of acrolein (3-HPMA) and crotonaldehyde (CR) (HPMMA), and tobacco alkaloids metabolites were measured by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) as previously described with slight modifications (293, 303). Briefly, 10 μ L of sample urine was combined with 440 μ L ammonium acetate (15 mM, pH 6.8) and 50 μ L internal standard and filtered through a 0.2 μ m PTFE membrane into a 2 mL UPLC vial. The samples were then applied to an ACQUITY UPLC core system (Acquity UPLC HSS T3 column (150 mm x 2.1 mm, 1.8 μ M)) and a Quattro Premier XE triple quadrupole MS coupled with an electrospray source (Waters Corporation). Ammonia acetate (15 mM, pH 6.8) and acetonitrile were used as mobile phase for chromatographic separation. The peak areas of analytes were quantified using at least 12 data points across the measurement peaks, and TargetLynx 4.1 (Waters Corporation) was used for peak integration, calibration, and quantification of analyze concentrations.

5. Tissue Collection and Storage

Immediately following the final exposure, mice were anesthetized with sodium pentobarbital (\approx 150 mg/kg, i.p.) followed by ventral thoracotomy and exsanguination via cardiac puncture for blood collection in EDTA-coated syringes. Whole blood was taken for complete blood cell count (CBC) analysis (50 μ L) and circulating angiogenic cell (CAC) analysis (300 μ L). The remaining blood was collected for additional tests or immediately centrifuged (4.6 rpm, 20 min, 4°C) to separate plasma; plasma was subsequently stored at -80°C for further analysis. Collected tissues (heart, lung, kidney, liver, pancreas, spleen, fat pad, and/or skeletal muscle) were frozen in liquid nitrogen and stored at -80°C for further analysis.

6. Complete Blood Cell Count (CBC)

Complete blood cell counts (CBCs) were measured (20 μ L blood) with a hematology analyzer calibrated with multispecies hematological reference controls (Hemavet 950FS; Drew Scientific, Inc.; Miami Lakes, FL) as described (151).

7. Plasma Biomarkers Quantification

Plasma total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, albumin, total protein, aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), and creatinine were measured on a Cobas Mira Plus clinical chemistry Autoanalyzer (Roche Diagnostics; Indianapolis, IN) as previously described (151) or on a Vet Axcel clinical chemistry system (Alfa Wassermann Diagnostic Technologies, LLC; West Caldwell, NJ).

8. Circulating Angiogenic Cell (CAC) Quantification

A 300 μ L sample of whole blood was lysed (3 mL BD PharmLyse, BD BioSciences, San Jose, CA; 10 min, RT), and after centrifugation (5 min, 500 \times g, RT), the supernatant was aspirated. The cell pellet was vortexed and resuspended in 1% BSA/PBS and divided into 2 equal fractions (i.e., isotype control or Flk-1/Sca-1 labeling). After another centrifugation (5 min, 500 \times g, RT), mononuclear cells were re-suspended in 1% BSA/PBS with 5 μ L anti-mouse CD16/CD32 Fc Block (0.5 μ g; BD Biosciences), and the samples incubated for on ice. After 10 minutes, 5 μ L of staining cocktail containing anti-Sca-1 (FITC; BD BioSciences) and anti-Flk-1 (APC, BD BioSciences) antibodies was added to each sample and the samples incubated on ice for 30 minutes. Cells were then washed with 1% BSA/PBS and centrifuged (5 min, 500 \times g, RT). After being resuspended

in 250 μ L of 1% BSA/PBS, 5 μ L of propidium iodide stock staining solution (ThermoFisher Scientific) was added to each tube, and cells were analyzed using a LSR II flow cytometer (BD BioSciences). Based on forward and side scatter, small non-debris events in a sub-lymphocyte population (3-5 μ m; sized using calibrated beads, Invitrogen, Carlsbad, CA, USA) were gated electronically and displayed in a two-color dot plot. Data were analyzed using FlowJo software (FlowJo LLC; Ashland, OR), and double positive events were normalized per 1 μ l of whole blood.

9. Leukocyte Subpopulation Quantification

A 100 μ L sample of whole blood was lysed (1 mL BD PharmLyse, BD BioSciences, San Jose, CA; 10 min, RT) and after centrifugation (5 min, 500 xg , RT), the supernatant was aspirated. The cell pellet was vortexed and resuspended with 1 mL 1% BSA/PBS and again centrifuged (5 min, 500 xg , RT), aspirated, and vortexed. This process was repeated a third time before the addition of 5 μ L anti-mouse CD16/CD32 Fc Block (50 μ g/mL; BD Biosciences) and incubation on ice. After 10 minutes, the cells were incubated on ice for 30 minutes with a cocktail containing FITC-anti-Nk1.1 (0.5 μ g), PE-anti-Ly6C (0.125 μ g), PerCPe710-anti-CD8 (0.125 μ g), PECy7-anti-CD62 (0.25 μ g), APC-anti-CD19 (0.125 μ g), Alexa 700-antiGr-1 (0.125 μ g), APCe780-anti-CD3 (1 μ g), eVolve605-CD11b (1 μ g), and e650-anti-CD4 (1 μ g). The stained cells were once again centrifuged (5 min, 500 xg , RT), aspirated, and vortexed before resuspension in 250 μ L 1% BSA/PBS before analysis on an LSR II flow cytometer (high speed, 90 seconds) (BD BioSciences). Cell numbers were analyzed using the FlowJo software (FlowJo LLC) and normalized per 1 μ l of whole blood. Monocytes double positive for Ly6C and CD62L were defined as a Ly6C^{high} subpopulation.

10. Statistical Analyses

Statistical analyses of flow cytometry data were performed using FlowJo V10 (FlowJo LLC; Ashland, OR) and SigmaPlot 12.5 (Systat Software, Inc.; San Jose, CA). Analyses of changes in body and tissue parameters as well as analyses of the results of CBC, plasma biomarker, and urine metabolite testing were performed using Excel 2016 (Microsoft; Redmond, WA) and SigmaPlot 12.5 (Systat Software, Inc.). Rank sum tests with Bonferroni's post-test were used to determine statistical significance when comparing two groups. Two-way ANOVA with Dunn's post-test was used when comparing multiple groups. Statistical significance assumed at $p < 0.05$ or as indicated. Data are expressed as the mean \pm SE or as indicated.

CHAPTER III

ASSOCIATIONS BETWEEN CIRCULATING ANGIOGENIC CELLS AND USE OF TRADITIONAL AND EMERGING TOBACCO PRODUCTS

A. Introduction

According to the World Health Organization, ischemic heart disease and stroke have been the leading causes of death worldwide since 2000, accounting for almost 27% of reported global deaths (2). In the United States, cardiovascular disease (CVD) constitutes a significant health and economic burden. The American Heart Association estimates that by 2035, more than 45% of the adult US population will have some form of CVD, and costs for medical care related to CVD will reach almost \$1 trillion (304). The development of CVD has been linked to the development of endothelium dysfunction; endothelium function is a balance of injury and repair, in part attributed to circulating angiogenic cells (CACs), wherein CAC levels are associated with endothelium health (62-66). Many studies have linked decreased levels of CACs with well-known CVD risk factors, including hypertension (70, 305), total (66) and low-density lipoprotein (LDL) (70, 306) cholesterol, diabetes (307, 308), flow-mediated brachial reactivity (66), and an increased Framingham Risk Score (66, 309, 310). Furthermore, levels of CACs have been shown to be decreased in individuals with more severe CVD (311-314), and Hill et al. (66)

have proposed that CACs may serve as a surrogate biomarker for vascular health and overall CVD risk.

Smoking has also been associated with CACs (315, 316). Smoking reduction and cessation have been linked to increased endothelium function attributed to greater levels or function of CACs (317-319), and specific components of traditional cigarettes and other tobacco products have been directly shown to alter levels of CACs (151, 320, 321). Although the effects of electronic cigarettes (e-cigs) on CACs are still debated (322-324), the many shared components between mainstream cigarette smoke (MCS) and e-cigs may contribute to changes in the number and function of CACs in a way that is likely to contribute to CVD development. Thus, the goal of this study was to examine the effects of personal exposures, specifically exposures to MCS and/or secondhand cigarette smoke (SHS) and e-cig aerosol, on hematological and endothelium-related changes in a human cohort.

B. Experimental Procedures

1. Study Population

Participants were recruited for the Louisville Healthy Heart Study (LHHS) as described in Chapter II, Section A.1.

2. Eligibility

Interested individuals were questioned regarding the eligibility requirements outlined in Chapter II, Section A.2. Individuals who met the eligibility requirements were enrolled in the study.

3. Case Definition

Study participants who were enrolled through the University of Louisville and

KentuckyOne Health hospitals cardiovascular clinic systems were defined as undergoing primary or secondary treatment based on the criteria outlined in Chapter II, Section A.3.

4. Recruitment

Details of participant recruitment are given in Chapter II, Section A.4.

5. Questionnaire

Each participant was administered a questionnaire as described in Chapter II, Section A.5. The questionnaire was designed to collect relevant information regarding the participant's demographics, medical history, and product use.

6. Biological Sample Collection and Processing

Upon confirming eligibility and obtaining written consent, blood and urine samples were collected, aliquoted, and stored as described in Chapter II, Section A.6.

7. Complete Blood Cell Count (CBC)

A sample of collected blood from the Vacutainer® K₂EDTA tube was used for a complete blood cell count (CBC) as described in Chapter II, Section A.7.

8. Circulating Angiogenic Cell (CAC) Quantification

Florescence-activated cell sorting (FACS) flow cytometry was used to analyze the number of CACs in blood samples collected in the Vacutainer® CPT Mononuclear Cell Preparation Tube as described in Chapter II, Section A.8. The gating scheme for this analysis is shown in Figure 3.1. The 15 CAC populations are described in Table 3.1.

9. Peripheral Blood Mononuclear Cell Aggregate (PMA) Quantification

FACS flow cytometry was used to analyze the number of platelet mononuclear cell aggregates (PMAs) in blood samples collected in the Vacutainer® ACD tube as described in Chapter II, Section A.9.

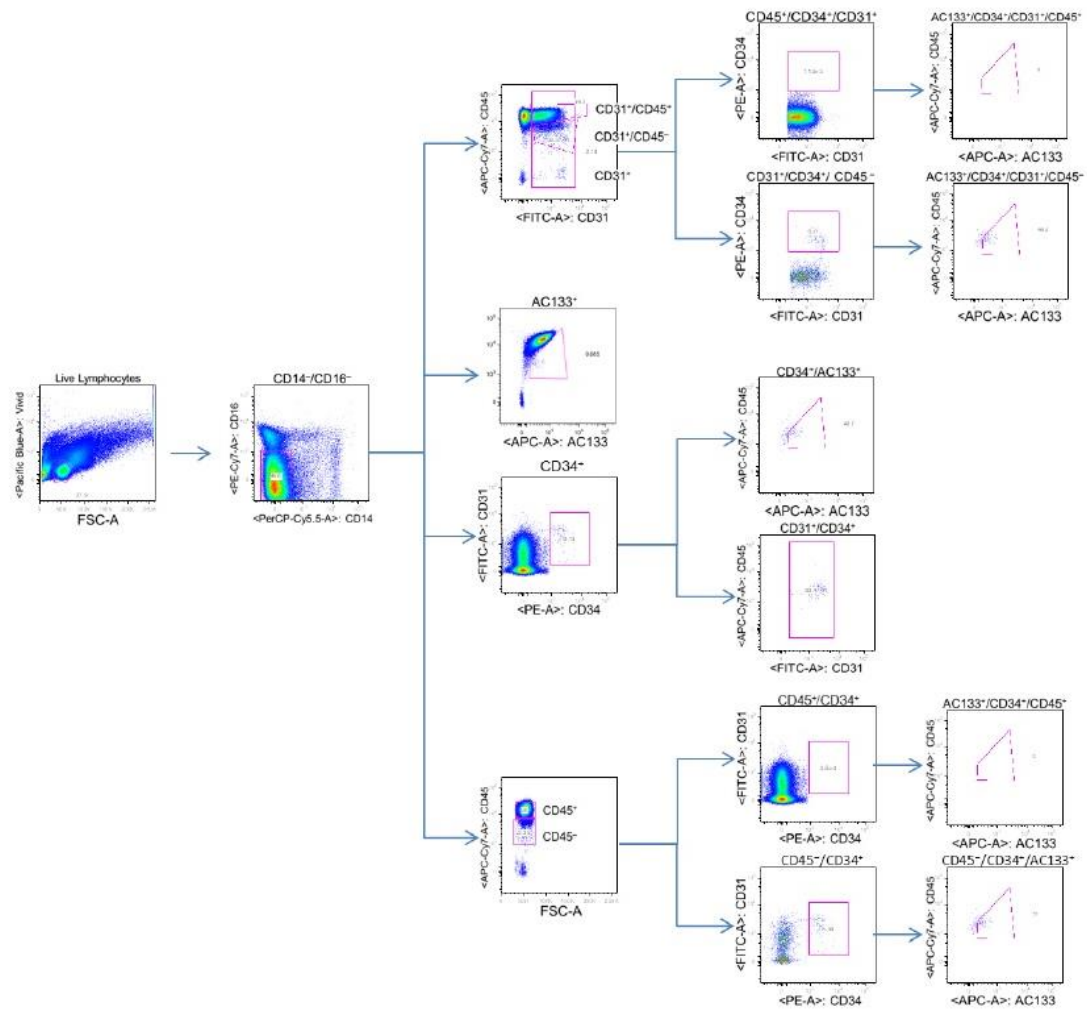


Fig. 3.1. Flow cytometry gating scheme for circulating angiogenic cell (CAC) populations. Abbr.: FSC, forward scatter.

Table 3.1. Antigenic identity of circulating angiogenic cells (CACs).

| Cell Group | Antigenic Marker | Cell Differentiation State |
|------------------|------------------|--|
| Stem | CAC-9 | CD34+ |
| | CAC-13 | CD34+/ACC133+ |
| | CAC-15 | CD34+/ACC133+/ CD45 ^{dim} |
| Early Progenitor | CAC-11 | AC133+ |
| | CAC-5 | AC133+/CD31+ |
| | CAC-4 | AC133+/CD34+/ CD31+/CD45+ |
| | CAC-14 | AC133+/CD34+/ CD45+ |
| Endothelial | CAC-10 | CD31+ |
| | CAC-6 | CD31+/CD34+ |
| | CAC-1 | CD31+/CD34+/ CD45 ^{dim} |
| | CAC-7 | CD31+/CD34+/ AC133-/CD45 ^{dim} |
| | CAC-3 | CD31+/CD34+/ AC133+/CD45 ^{dim} |
| Hematopoietic | CAC-12 | CD45+ |
| | CAC-2 | CD45+/CD34+/ CD31+ |
| | CAC-8 | CD45+/CD34+/ CD31+/AC133- |

10. Urine Metabolite Quantification

Collected random catch urine samples were used to quantify levels of creatinine and volatile organic compound (VOC) and tobacco alkaloids metabolites using analytical methods as described in Chapter II, Section A.10.

11. Statistical Analyses

Differences between demographics, measures of systemic injury, urinary metabolites, and CACs were assessed as described in Chapter II, Section A.11.

C. Results

1. Baseline Characteristics by Tobacco Product Use Categories

Overall, the mean (SE) age of the Louisville Healthy Heart study population was 49 (± 1) years, and the majority of participants were women (66%) (Table 3.2). E-cig or dual users were significantly younger than never or former smokers and cigarette only smokers (Table 3.2). There were not notable sex differences, with similar levels of cigarette only and e-cig or dual use between males and females, and both cigarette only and e-cig or dual users were predominately white (68% and 71%, respectively), although these differences were not statistically significant (Table 3.2). Current nonusers of tobacco were more educated and had higher annual income than current users of tobacco products (Table 3.2). Never smokers reported higher prevalence of high blood cholesterol and hypertension in their family history, while former smokers reported a lower family prevalence of diabetes (Table 3.2). Current tobacco product users reported more secondhand smoke exposure, but reports of alcohol consumption and regular exercise were similar between all use groups (Table 3.2).

Table 3.2. Demographic characteristics by tobacco product use categories.

| Continuous Variables – mean (SE) | Total (n=143) | Never Smokers (n=62) | Former Smokers (n=25) | CIG Only (n=28) | E-Cig or Dual (n=28) | p-value |
|---|----------------------|-----------------------------|------------------------------|------------------------|-----------------------------|------------------|
| Age | 49 (1) | 53 (2) | 53 (2) | 49 (2) | 40 (1) | <0.001 |
| BMI | 30 (1) | 30 (1) | 30 (1) | 29 (1) | 30 (1) | 0.263 |
| Categorical Variables – n (%) | Total | Never Smokers | Former Smokers | CIG Only | E-Cig or Dual | p-value |
| Sex | | | | | | 0.065 |
| Female | 95 (66) | 47 (76) | 18 (72) | 14 (50) | 16 (57) | |
| Male | 48 (34) | 15 (24) | 7 (28) | 14 (50) | 12 (43) | |
| Race | | | | | | 0.363 |
| Caucasian | 103 (72) | 44 (71) | 20 (80) | 19 (68) | 20 (71) | |
| African American | 31 (22) | 11 (18) | 4 (16) | 9 (32) | 7 (25) | |
| Other | 9 (6) | 7 (11) | 1 (4) | 0 (0) | 1 (4) | |
| Family Medical History | | | | | | 0.126 |
| Diabetes | 114 (80) | 54 (87) | 17 (68) | 23 (82) | 20 (71) | 0.014 |
| High Blood Cholesterol | 66 (46) | 33 (53) | 6 (24) | 12 (43) | 15 (54) | 0.025 |
| Hypertension | 50 (35) | 29 (47) | 8 (32) | 9 (32) | 4 (14) | 0.015 |
| Premature CVD | 96 (67) | 47 (76) | 17 (68) | 18 (64) | 14 (50) | 0.028 |
| Lifestyle | | | | | | |
| Secondhand Smoke | 38 (27) | 13 (21) | 4 (16) | 11 (39) | 10 (36) | <0.001 |
| Regular Exercise | 40 (28) | 7 (11) | 1 (4) | 12 (43) | 20 (71) | 0.875 |
| Alcohol Consumption | 84 (59) | 38 (62) | 15 (60) | 16 (57) | 15 (54) | 0.376 |
| Education | | | | | | <0.001 |
| < HS | 80 (56) | 35 (56) | 11 (44) | 4 (14) | 5 (18) | |
| Some HS/HS Grad | 44 (31) | 10 (16) | 5 (20) | 15 (54) | 14 (50) | |
| Some College | 22 (15) | 6 (10) | 4 (16) | 5 (18) | 7 (25) | |
| 2-4 Year Degree | 9 (6) | 2 (3) | 3 (12) | 3 (11) | 1 (4) | |
| Masters/Doctorate | 17 (12) | 13 (21) | 2 (8) | 1 (4) | 1 (4) | |

| | | | | | | |
|--------------------------------|---------|---------|---------|---------|---------|--------------|
| Annual Household Income | | | | | | 0.019 |
| <\$20,000 | 18 (13) | 5 (8) | 2 (8) | 5 (18) | 6 (21) | |
| \$20,000-\$45,000 | 37 (26) | 10 (16) | 5 (20) | 11 (39) | 11 (39) | |
| \$45,000-\$89,999 | 47 (33) | 21 (34) | 11 (44) | 8 (29) | 7 (25) | |
| > \$90,000 | 41 (29) | 26 (42) | 7 (28) | 4 (14) | 4 (14) | |

Values = mean ± SE. **Abbr.:** **BMI**, body mass index; **CIG**, cigarette; **CVD**, cardiovascular disease; **E-cig**, electronic cigarette; **HS**, high school.

Two-thirds of participants (n=94, 66%) reported some form of CVD history, with almost half of those individuals reporting a diagnosis of hypertension (n=69, 49%) and approximately one-third reporting a diagnosis of high blood cholesterol (n=47, 33%) (Table 3.3). Additionally, over half of the participants reported taking some form of CVD medication (n=76, 53%) (Table 3.3). By tobacco product use category, fewer e-cig or dual users reported significant CVD history than both never and former smokers as well as less use of CVD medication than all other use categories (Table 3.3). E-cig or dual users were also less likely to have high blood cholesterol and hypertension than all other use categories and to use aspirin or beta-blockers than former smokers, while never smokers were less likely to have hypertension and to use beta blockers than former smokers (Table 3.3). Former smokers and cigarette only users showed similar likelihoods of having some form of CVD and using CVD medication (Table 3.3).

2. Tobacco Product Use Categories and Hematological Measures

E-cig or dual users had significantly higher levels of leukocytes than never smokers, driven by a significant increase in granulocytes, as well as an increased level of hemoglobin (Table 3.4). Cigarette only users had an increased red cell distribution width compared with both never smokers and e-cig or dual users (Table 3.4). Although platelets levels (measured by CBC) were unchanged between the groups, platelet monocyte aggregates (PMAs; measured by flow cytometry) were significantly different between the categories, with e-cig or dual users showing significantly lower levels of PMAs compared with never smokers, former smokers, and cigarette only users (Table 3.4). Never smokers and former smokers showed no significant differences between any of the hematological measures measured by CBC (Table 3.4).

Table 3.3. Medical history and medication use by tobacco product use categories.

| Categorical Variables – n (%) | Total (n=143) | Never Smokers (n=62) | Former Smokers (n=25) | CIG Only (n=28) | E-Cig or Dual (n=28) | p-value |
|---|--------------------------|-------------------------------------|--------------------------------------|----------------------------|-------------------------------------|------------------|
| Medical History | | | | | | |
| <i>CVD History</i> | 94 (66) | 44 (71) | 21 (84) | 18 (64) | 11 (39) | 0.005 |
| Angina | 13 (9) | 4 (7) | 5 (20) | 4 (14) | 0 (0) | 0.055 |
| Atrial Fibrillation/Flutter | 8 (6) | 4 (7) | 2 (8) | 2 (7) | 0 (0) | 0.793 |
| Blood Clot | 7 (5) | 2 (3) | 3 (12) | 2 (7) | 0 (0) | 0.135 |
| CABG/PCI/Stents | 15 (11) | 6 (10) | 3 (12) | 4 (14) | 2 (7) | 0.651 |
| Heart Failure | 9 (6) | 1 (2) | 4 (16) | 4 (15) | 0 (0) | 0.004 |
| Heart Valve Disease | 5 (4) | 2 (3) | 2 (8) | 1 (4) | 0 (0) | 0.508 |
| High Blood Cholesterol | 47 (33) | 22 (36) | 12 (48) | 9 (32) | 4 (14) | 0.034 |
| Hypertension | 69 (49) | 31 (50) | 20 (80) | 14 (52) | 4 (14) | <0.001 |
| Heart Attack | 11 (8) | 4 (7) | 3 (12) | 4 (14) | 0 (0) | 0.126 |
| Peripheral Artery Disease | 5 (4) | 3 (5) | 2 (8) | 0 (0) | 0 (0) | 0.246 |
| Stroke | 7 (5) | 2 (3) | 1 (4) | 2 (7) | 2 (7) | 0.809 |
| <i>Other Significant Medical Condition(s)</i> | 39 (27) | 21 (34) | 7 (28) | 4 (14) | 7 (25) | 0.288 |
| Diabetes | 28 (20) | 13 (21) | 8 (32) | 4 (14) | 3 (11) | 0.234 |
| BMI \geq 30 | 64 (45) | 26 (42) | 11 (44) | 11 (39) | 16 (57) | 0.374 |
| Medication Use | | | | | | |
| <i>CVD Medication</i> | 76 (53) | 38 (61) | 18 (72) | 15 (54) | 5 (18) | <0.001 |
| ACE Inhibitor | 26 (19) | 12 (20) | 6 (24) | 7 (27) | 1 (4) | 0.122 |
| Angiotensin-receptor Blocker | 12 (9) | 4 (7) | 4 (16) | 4 (15) | 0 (0) | 0.147 |
| Aspirin | 33 (24) | 13 (22) | 11 (44) | 8 (31) | 1 (4) | 0.006 |
| Beta-blocker | 30 (22) | 10 (17) | 12 (48) | 8 (31) | 0 (0) | <0.001 |
| Calcium-channel Blocker | 16 (12) | 7 (12) | 5 (20) | 4 (15) | 0 (0) | 0.144 |
| Diuretics | 28 (21) | 11 (18) | 8 (32) | 7 (27) | 2 (8) | 0.196 |
| Statins | 31 (22) | 13 (21) | 9 (36) | 7 (25) | 2 (7) | 0.070 |

| | | | | | | |
|-------------------------|----------|---------|---------|---------|---------|-------|
| <i>Other Medication</i> | 120 (84) | 54 (87) | 23 (92) | 24 (86) | 19 (68) | 0.099 |
| Oral Contraceptives | 49 (34) | 16 (26) | 7 (28) | 14 (50) | 12 (43) | 0.091 |

Values = mean \pm SE. **Abbr.:** **ACE**, angiotensin-converting enzyme; **BMI**, body mass index; **CABG**, coronary artery bypass grafting; **CIG**, cigarette; **CVD**, cardiovascular disease; **E-cig**, electronic cigarette; **PCI**, percutaneous coronary intervention.

Table 3.4. Hematological measures by tobacco product use categories.

| Complete Blood Cell Count – mean ± SE | Total (n=139) | Never Smokers (n=61) | Former Smokers (n=24) | CIG Only (n=27) | E-Cig or Dual (n=27) | p-value |
|---|----------------------|-------------------------------------|--------------------------------------|----------------------------|---------------------------------|------------------|
| White Blood Cell (K/ μ L) | 6.43 ± 0.17 | 5.82 ± 0.20 | 6.10 ± 0.37 | 7.05 ± 0.42 | 7.49 ± 0.42 | 0.002 |
| Lymphocytes (%) | 30.89 ± 0.62 | 32.17 ± 1.02 | 30.19 ± 1.10 | 31.81 ± 1.52 | 27.68 ± 1.19 | 0.098 |
| Monocytes (%) | 6.90 ± 0.16 | 7.12 ± 0.25 | 7.46 ± 0.33 | 6.41 ± 0.35 | 6.41 ± 0.42 | 0.049 |
| Granulocytes (%) | 62.27 ± 0.65 | 60.49 ± 1.05 | 63.21 ± 0.93 | 61.78 ± 1.58 | 65.93 ± 1.37 | 0.016 |
| Red Blood Cell (M/ μ L) | 4.68 ± 0.05 | 4.6 ± 0.06 | 4.68 ± 0.12 | 4.52 ± 0.09 | 4.92 ± 0.14 | 0.107 |
| Hemoglobin (g/dL) | 14.1 ± 0.1 | 13.8 ± 0.2 | 13.9 ± 0.3 | 13.9 ± 0.4 | 15.0 ± 0.4 | 0.029 |
| Hematocrit (%) | 42.0 ± 0.5 | 41.4 ± 0.5 | 41.7 ± 1.0 | 41.3 ± 1.2 | 44.4 ± 1.3 | 0.171 |
| Mean Corpuscular Volume (fL) | 89.8 ± 0.5 | 89.2 ± 0.6 | 89.3 ± 0.9 | 91.1 ± 1.6 | 90.2 ± 1.1 | 0.621 |
| Mean Corpuscular Hemoglobin (pg) | 30.3 ± 0.3 | 29.8 ± 0.3 | 30.0 ± 0.5 | 30.9 ± 0.8 | 30.9 ± 1.0 | 0.534 |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 33.7 ± 0.3 | 33.4 ± 0.2 | 33.6 ± 0.5 | 34.0 ± 0.8 | 34.3 ± 1.1 | 0.831 |
| Red Cell Distribution Width (%) | 14.8 ± 0.1 | 14.5 ± 0.1 | 15.0 ± 0.3 | 15.6 ± 0.4 | 14.2 ± 0.4 | 0.008 |
| Platelets (K/ μ L) | 263 ± 6 | 256 ± 8 | 270 ± 16 | 259 ± 16 | 276 ± 13 | 0.551 |
| PMAAs (%) | 23.3 ± 0.8 | 23.9 ± 1.1 | 22.8 ± 1.8 | 23.7 ± 2.2 | 21.6 ± 1.7 | <0.001 |
| Mean Platelet Volume (fL) | 8.4 ± 0.1 | 8.3 ± 0.2 | 8.7 ± 0.4 | 8.4 ± 0.3 | 8.4 ± 0.2 | 0.787 |

Values = mean ± SE. **Abbr.:** CIG, cigarette; **E-cig**, electronic cigarette; **PMAAs**, platelet mononuclear cell aggregates.

3. Tobacco Products Use Categories and Urinary VOC Metabolites

Analysis of urinary VOC and tobacco alkaloid metabolites showed that never and former smokers had lower mean levels of most VOC and tobacco alkaloid metabolites compared with current users of tobacco products (Table 3.5). Never smokers and former smokers showed no significantly different levels of measured VOC or tobacco alkaloid metabolites (Table 3.5). All measured metabolites except N-acetyl-S-(benzyl)-L-cysteine (BMA) showed significant differences between the tobacco product use categories, and the majority of VOC metabolites were higher in cigarette only users and/or e-cig or dual users than in never or former smokers (Table 3.5). Almost one-third of the VOC metabolites were significantly different between cigarette only users and e-cig or dual users, with levels of cyanoethyl mercapturic acid (CEMA), 3-hydroxypropylmercapturic acid (3HPMA), N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA), N-acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine (3MHBMA), N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine (HPMMA), N-acetyl-S-(N-methylcarbamoyl)-L-cysteine (AMCC), and anatabine (ANTB) significantly higher in cigarette only users compared with e-cig or dual users (Table 3.5). Interestingly, while never smokers showed significantly lower levels of AMCC, N-acetyl-S-(2-hydroxypropyl)-L-cysteine (2HPMA), 2-methylhippuric acid (2MHA), and 3-methylhippuric acid + 4-methylhippuric acid (3MHA + 4MHA), compared with both cigarette only users and e-cig or dual users, levels of these metabolites in former smokers were not significantly different compared with e-cig or dual users (Table 3.5). Additionally, former smokers had levels of anabasine (ANB) that were not significantly different from either e-cig or dual users or cigarette only users, although levels were also not significantly different from those found in never smokers (Table 3.5). Unsurprisingly,

Table 3.5. Mean urinary volatile organic compound (VOC) metabolite levels by tobacco product use categories (ng/mg).

| Parent Compound | Analyte | Total (n=103) | Never Smokers (n=43) | Former Smokers (n=17) | CIG Only (n=23) | E-Cig or Dual (n=20) | p-value |
|-----------------------|-------------|-----------------|----------------------|-----------------------|-----------------|----------------------|---------|
| Acrolein | CEMA | 167.8 ± 17.3 | 72.0 ± 14.0 | 71.4 ± 13.6 | 365.0 ± 39.7 | 228.9 ± 33.6 | <0.001 |
| | 3HPMA | 822.8 ± 107.6 | 313.8 ± 67.5 | 267.1 ± 38.7 | 1,837.9 ± 280.6 | 1,222.0 ± 280.7 | <0.001 |
| Acrylamide | AAMA | 103.9 ± 8.7 | 67.8 ± 6.0 | 69.0 ± 10.5 | 192.3 ± 25.9 | 109.5 ± 16.0 | <0.001 |
| 1,3-Butadiene | DHBMA | 422.0 ± 20.1 | 343.4 ± 17.3 | 410.9 ± 53.2 | 573.0 ± 56.5 | 427.0 ± 37.3 | 0.001 |
| | 3MHBMA | 25.1 ± 3.5 | 5.8 ± 0.6 | 4.6 ± 0.5 | 65.7 ± 8.6 | 37.5 ± 7.9 | <0.001 |
| Crotonaldehyde | HPMMA | 606.3 ± 70.5 | 240.3 ± 32.3 | 256.6 ± 50.5 | 1,347.0 ± 185.2 | 838.7 ± 168.2 | <0.001 |
| N,N-Dimethylformamide | AMCC | 284.8 ± 22.3 | 154.2 ± 13.2 | 227.5 ± 34.0 | 526.1 ± 52.9 | 336.8 ± 51.5 | <0.001 |
| Ethylbenzene, styrene | PGA | 290.6 ± 15.5 | 234.0 ± 17.2 | 220.2 ± 27.7 | 407.9 ± 36.5 | 337.2 ± 37.0 | <0.001 |
| Propylene Oxide | 2HPMA | 61.8 ± 8.8 | 50.3 ± 17.8 | 57.4 ± 21.8 | 87.9 ± 10.0 | 60.3 ± 10.4 | <0.001 |
| Styrene | MA | 261.0 ± 16.5 | 171.9 ± 14.9 | 180.0 ± 21.0 | 391.5 ± 40.2 | 371.6 ± 33.8 | <0.001 |
| Toluene | BMA | 15.1 ± 2.1 | 15.2 ± 3.2 | 11.7 ± 3.4 | 18.1 ± 6.1 | 14.5 ± 3.7 | 0.752 |
| Xylene | 2MHA | 56.4 ± 7.7 | 22.2 ± 4.5 | 41.8 ± 13.4 | 131.6 ± 24.5 | 56.0 ± 12.0 | <0.001 |
| | 3MHA + 4MHA | 403.9 ± 46.1 | 250.1 ± 76.3 | 275.9 ± 85.3 | 725.5 ± 90.2 | 473.5 ± 72.3 | <0.001 |
| Anabasine | ANB | 4.7 ± 0.4 | 2.8 ± 0.3 | 4.5 ± 1.1 | 7.5 ± 1.0 | 5.6 ± 0.9 | <0.001 |
| Anatabine | ANTB | 3.1 ± 0.5 | 0.8 ± 0.1 | 1.1 ± 0.3 | 8.1 ± 1.5 | 4.0 ± 1.4 | <0.001 |
| Nicotine | NIC | 657.2 ± 130.4 | 11.3 ± 2.4 | 13.9 ± 2.9 | 1,350.4 ± 287.6 | 1,795.5 ± 443.8 | <0.001 |
| | COT | 527.0 ± 91.6 | 2.3 ± 0.3 | 2.6 ± 0.6 | 1,413.8 ± 242.4 | 1,081.3 ± 215.7 | <0.001 |
| | 3HC | 1,716.0 ± 258.2 | 9.5 ± 3.1 | 11.7 ± 3.7 | 4,234.2 ± 453.8 | 3,937.7 ± 682.6 | <0.001 |

Values = mean ± SE. **Abbr.:** AAMA, N-acetyl-S-(2-carbamoyl-ethyl)-L-cysteine; AMCC, N-acetyl-S-(N-methylcarbamoyl)-L-

cysteine; **ANB**, anabasine; **ANTB**, anatabine; **BMA**, N-acetyl-S-(benzyl)-L-cysteine; **CEMA**, N-acetyl-S-(2-carboxyethyl)-L-cysteine; **CIG**, cigarette; **COT**, cotinine; **DHBMA**, N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine; **E-cig**, electronic cigarette; **3HC**, trans-3'-hydroxycotinine; **2HPMA**, N-acetyl-S-(2-hydroxypropyl)-L-cysteine; **3HPMA**, N-acetyl-S-(3-hydroxypropyl)-L-cysteine; **HPMMA**, N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine; **MA**, mandelic acid; **2MHA**, 2-methylhippuric acid; **3MHA**, 3-methylhippuric acid; **4MHA**, 4-methylhippuric acid; **3MHBMA**, N-acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine; **NIC**, nicotine; **PGA**, phenylglyoxylic acid.

metabolites of tobacco alkaloids (nicotine (NIC), cotinine (COT), and trans-3'-hydroxycotinine (3HC)) were significantly increased in cigarette only users and e-cig or dual users compared with never or former smokers, but levels were not significantly different between the two current product use categories (Table 3.5).

4. Tobacco Product Use Categories and CACs

In the fully adjusted (adjusted for age, sex, CVD history, use of CVD medications, family history of CVD, SHS exposure, and SES (income category used as proxy indicator)) multivariable-linear regression model testing the association between tobacco product use categories and log-transformed CAC levels, levels of CAC-12 (CD45+) were significantly decreased in e-cig or dual users compared with never smokers (-6.6%; $p=0.008$) (Table 3.6). There were no significant differences in the levels of the other fourteen measured CAC populations between any of the tobacco product use categories (Table 3.6). In order to explore dimension reduction of the demographic adjustments used in the fully adjusted model, a random forest model was used to select the demographic variables that varied the most across the four tobacco product use categories independently of smoking status. Based on the resulting variable importance plot (Fig. 3.3), multivariable-adjusted linear regression models were again used to assess the relationship between log-transformed CAC measures and tobacco product use categories adjusting for age and SES. When the model was adjusted only for age and SES, levels of CAC-12 (CD45+) remained decreased in e-cig or dual users compared with never smokers (-6.4%; $p=0.004$), and CAC-3 (CD31+/CD34+/AC133+/CD45_{dim}) was also found to be significantly different between never smokers and e-cig or dual users, with e-cig or dual users showing significantly increased levels (+120%; $p=0.03$) (Table 3.7, Fig. 3.3).

Table 3.6. Percent change in levels of circulating angiogenic cells (CACs) comparing tobacco product use categories with never smokers in the fully adjusted model.

| CAC | Former Smoker, % (95% CI) | CIG Only, % (95% CI) | E-cig or Dual User, % (95% CI) |
|---|------------------------------|-------------------------|-----------------------------------|
| CAC-1 (CD31+/CD34+/CD45 _{dim}) | 15.5 (-20.2 to 67.0) | -5.8 (-33.2 to 32.8) | 4.3 (-30.7 to 56.9) |
| CAC-2 (CD45+/CD34+/CD31+) | 2.3 (-29.9 to 49.4) | -1.3 (-30.6 to 40.4) | -15.4 (-44.3 to 28.6) |
| CAC-3 (CD31+/CD34+/AC133+/CD45 _{dim}) | 44.0 (-29.0 to 192.1) | 8.0 (-44.1 to 108.6) | 75.8 (-19.6 to 284.4) |
| CAC-4 (AC133+/CD34+/CD31+/CD45+) | -15.9 (-50.0 to 41.2) | -14.9 (-47.5 to 37.9) | -7.0 (-47.6 to 65.0) |
| CAC-5 (AC133+/CD31+) | 8.0 (-32.6 to 73.1) | -17.3 (-46.7 to 28.2) | -25.9 (-56.0 to 24.8) |
| CAC-6 (CD31+/CD34+) | -3.9 (-40.0 to 54.0) | -20.0 (-48.4 to 24.1) | -30.2 (-58.6 to 17.6) |
| CAC-7 (CD31+/CD34+/AC133-/CD45 _{dim}) | 13.9 (-21.2 to 64.7) | -5.9 (-33.2 to 32.6) | 1.9 (-32.2 to 53.2) |
| CAC-8 (CD45+/CD34+/CD31+/AC133-) | 5.1 (-27.9 to 53.2) | 0.9 (-29.0 to 43.2) | -16.1 (-44.7 to 27.3) |
| CAC-9 (CD34+) | -6.3 (-32.8 to 30.6) | -8.8 (-33.0 to 24.2) | -7.9 (-36.2 to 32.9) |
| CAC-10 (CD31+) | 17.8 (-10.5 to 55.1) | 8.3 (-16.1 to 39.8) | -8.7 (-32.6 to 23.7) |
| CAC-11 (AC133+) | 25.3 (-13.7 to 81.9) | -1.9 (-30.6 to 38.8) | -24.1 (-49.8 to 14.7) |
| CAC-12 (CD45+) | 1.3 (-3.1 to 5.9) | -2.8 (-6.7 to 1.3) | -6.6 (-11.1 to -1.9) |
| CAC-13 (CD34+/ACC133+) | -6.0 (-38.5 to 43.7) | -0.3 (-32.8 to 48.0) | 18.8 (-25.7 to 89.9) |
| CAC-14 (AC133+/CD34+/CD45+) | -10.0 (-40.3 to 35.7) | -12.5 (-40.2 to 28.2) | 14.7 (-27.2 to 80.6) |
| CAC-15 (CD34+/ACC133+/CD45 _{dim}) | 10.6 (-28.9 to 72.1) | -3.0 (-35.7 to 46.4) | 26.5 (-22.5 to 106.4) |

Estimates were derived using the formula $(e^{\beta} - 1) \times 100\%$, where β represents the β -coefficient of the multivariable-adjusted association between product use categories and ln (CAC levels). Models adjusted for age, sex, cardiovascular disease history, use of cardiovascular medications, family history of CVD, secondhand smoke exposure, and socioeconomic status (income category used as proxy indicator). Bolded values are statistically significant at $p < 0.05$. **Abbr.:** **CAC**, circulating angiogenic cell; **CI**, confidence interval; **CIG**, cigarette; **E-cig**, electronic cigarette.

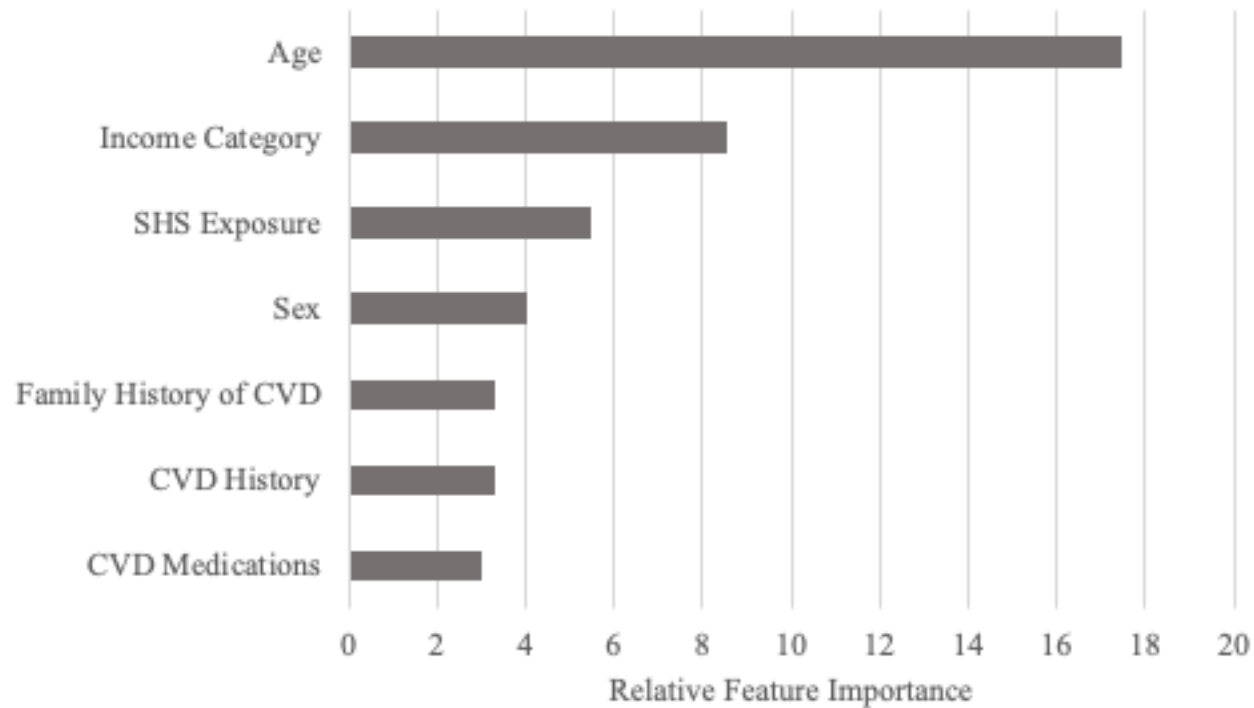


Fig. 3.2. Relative feature importance of demographic variables on levels of circulating angiogenic cells (CACs). A random forest model was used to determine the most important demographic variables influencing levels of CACs independently of tobacco product use category. Importance was calculated by reduction of gini impurity at relevant branchings. **Abbr.:** CAC, circulating angiogenic cell; CVD, cardiovascular disease; SHS, secondhand smoke.

Table 3.7. Percent change in levels of circulating angiogenic cells (CACs) comparing tobacco product use categories to never smokers in the secondary adjusted model.

| CAC | Former Smoker, % (95% CI) | CIG Only, % (95% CI) | E-cig or Dual User, % (95% CI) |
|---|------------------------------|-------------------------|-----------------------------------|
| CAC-1 (CD31+/CD34+/CD45 _{dim}) | 23.7 (-13.3 to 76.4) | -4.6 (-31.4 to 32.8) | 10.8 (-22.6 to 58.7) |
| CAC-2 (CD45+/CD34+/CD31+) | 4.7 (-26.8 to 49.9) | -1.4 (-29.4 to 37.7) | -13.3 (-39.7 to 24.6) |
| CAC-3 (CD31+/CD34+/AC133+/CD45 _{dim}) | 38.5 (-29.8 to 173.2) | 24.5 (-33.8 to 134.2) | 120.3 (10.8 to 337.8) |
| CAC-4 (AC133+/CD34+/CD31+/CD45+) | -20.2 (-51.2 to 30.6) | -8.3 (-42.0 to 44.9) | 11.3 (-32.4 to 83.1) |
| CAC-5 (AC133+/CD31+) | 7.2 (-31.5 to 67.8) | -15.8 (-44.5 to 27.7) | -19.1 (-48.6 to 27.2) |
| CAC-6 (CD31+/CD34+) | 0.6 (-35.6 to 57.4) | -18.9 (-46.5 to 23.0) | -28.0 (-54.2 to 13.2) |
| CAC-7 (CD31+/CD34+/AC133-/CD45 _{dim}) | 23.1 (-13.7 to 75.7) | -5.7 (-32.3 to 31.2) | 6.9 (-25.4 to 53.1) |
| CAC-8 (CD45+/CD34+/CD31+/AC133-) | 8.6 (-24.1 to 55.3) | 0.0 (-28.3 to 39.5) | -15.1 (-40.9 to 21.9) |
| CAC-9 (CD34+) | -3.9 (-29.9 to 31.7) | -8.9 (-32.0 to 22.2) | -8.8 (-33.7 to 25.5) |
| CAC-10 (CD31+) | 19.2 (-8.4 to 55.1) | 9.1 (-14.6 to 39.4) | -2.2 (-25.1 to 27.6) |
| CAC-11 (AC133+) | 21.5 (-15.0 to 73.6) | 0.7 (-27.7 to 40.4) | -17.4 (-42.4 to 18.5) |
| CAC-12 (CD45+) | 0.5 (-3.7 to 4.9) | -2.4 (-6.2 to 1.5) | -6.4 (-10.3 to -2.2) |
| CAC-13 (CD34+/ACC133+) | -7.0 (-38.1 to 39.5) | 5.4 (-27.8 to 53.7) | 35.5 (-10.1 to 104.3) |
| CAC-14 (AC133+/CD34+/CD45+) | -13.6 (-41.5 to 27.4) | -9.8 (-37.2 to 29.5) | 25.4 (-15.4 to 85.8) |
| CAC-15 (CD34+/ACC133+/CD45 _{dim}) | 10.0 (-28.2 to 68.5) | 3.6 (-30.3 to 54.1) | 44.8 (-5.9 to 122.9) |

Estimates were derived using the formula $(e^{\beta} - 1) \times 100\%$, where β represents the β -coefficient of the multivariable-adjusted association between product use categories and ln (CAC levels). Models adjusted for age and socioeconomic status (income category used as proxy indicator). Bolded values are statistically significant at $p < 0.05$. **Abbr.:** **CAC**, circulating angiogenic cell; **CI**, confidence interval; **CIG**, cigarette; **E-cig**, electronic cigarette.

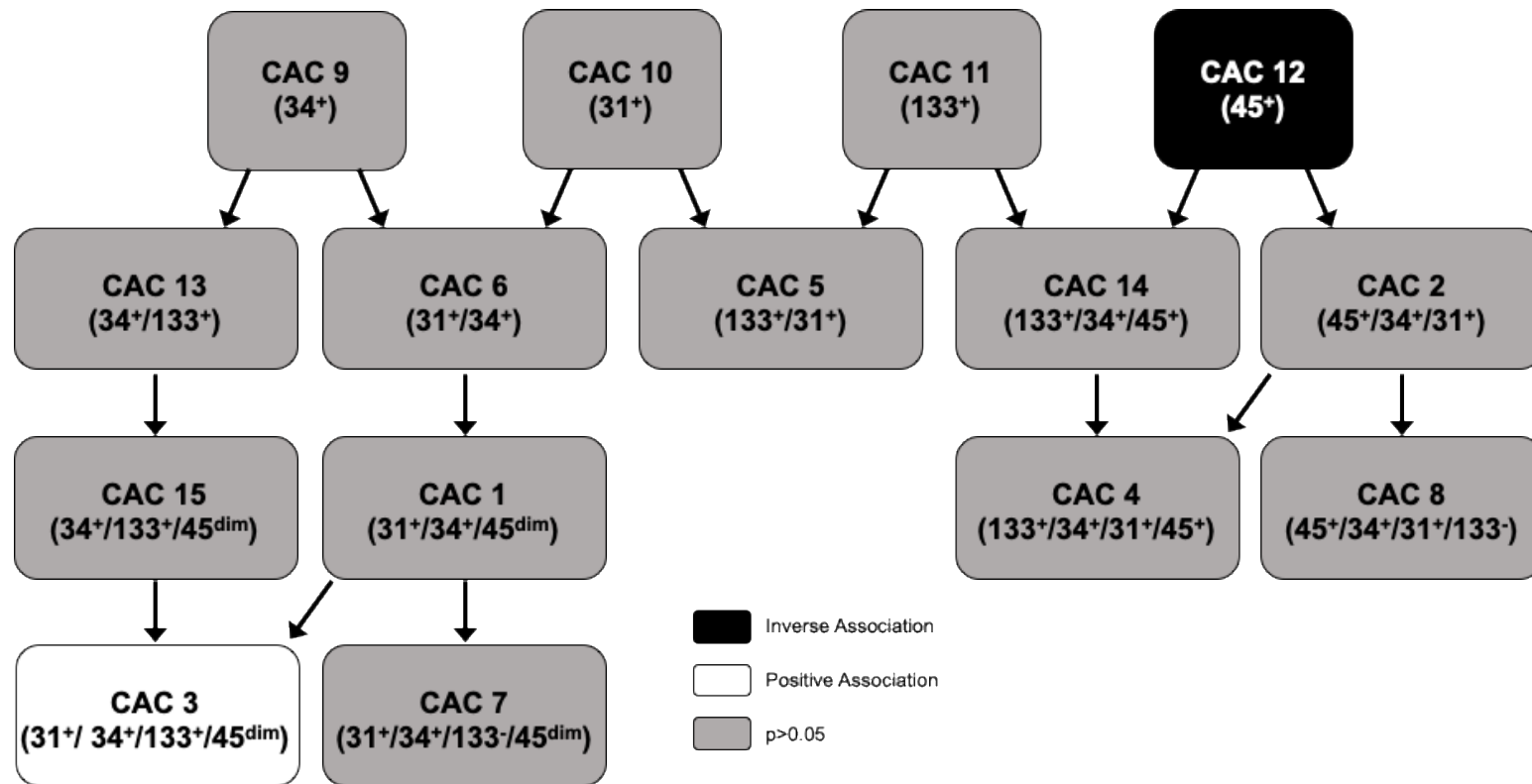


Fig. 3.3. Interrelationships between circulating angiogenic cells (CACs) associated with e-cig or dual use compared with never smokers. Levels of 15 antigenetically defined populations of CACs were measured in 103 participants and examined for their association with tobacco product use categories adjusted for age and socioeconomic status. Associations were only found between e-cig or dual users and CAC-3 (31⁺/34⁺/133⁺/45^{dim}) and CAC-12 (45⁺) compared with levels of CACs measured in never smokers.

D. Discussion

The Louisville Healthy Heart Study represents one of the first human cohort studies to examine the associations between a large number of CAC populations, urinary VOC and tobacco alkaloid metabolites, and specific types of tobacco product use, including e-cig or dual e-cig and traditional cigarette users. Although many adults are using e-cigs as an alternative to traditional cigarettes (325), there has been a marked increase in e-cig use among youths (83, 182, 183). Although the current study cohort did not include any individuals under the age of 18 (based on exclusion criteria), this trend in the use of e-cig devices is reflected in our cohort, as e-cig or dual users were significantly younger than never and former smokers and cigarette only users. The use of e-cigs by younger individuals—and especially individuals who may not have previously been using tobacco products—is a cause for concern as e-cigs have the potential to create a new generation of individuals addicted to nicotine (326). The current cohort also contained a greater number of females than males (66% vs 34%), although this ratio was not statistically significant. As males are more likely than women to be smokers (83), there may exist relationships between tobacco product users and the measured outcomes that were not noted due to the imbalance of sex in the participants in the current cohort. Use of tobacco products has also previously been associated with lower education levels and lower household incomes, as was seen in our cohort (83).

Likely due to their younger age, e-cig or dual users were found to have a lower prevalence of high blood cholesterol and hypertension than never smokers, former smokers, or cigarette only users and to have a lower usage of CVD medicines. Increased blood pressure and levels of cholesterol are known to increase with age (327, 328), so it is

unsurprising that the youngest participants on average in this study have less prevalence of major cardiovascular conditions. Perhaps also unsurprising is the significantly lower prevalence of hypertension in never smokers than in former smokers as well as the lower use of beta-blockers, which are generally prescribed to help lower blood pressure. Although several epidemiological studies have paradoxically shown decreased blood pressure in smokers compared with nonsmokers or former smokers (329-332), smoking is still considered a risk factor for hypertension (333-335). However, there is evidence associating previous smoking with increased blood pressure, as former smokers have been shown to have increased blood pressure and hypertension compared with never smokers (331, 336). This may be due to the development of arterial stiffness, which is closely linked with the development of hypertension (337) and which has been shown to be exacerbated by smoking (338-341). Although this increased arterial stiffness has been shown to be reversible, it may take more than a decade to achieve levels of stiffness similar to those found in never smokers (342). This persistent stiffness even after smoking cessation may be an important factor in the lower prevalence of hypertension seen in our never-smoking participants compared with those who had formerly smoked.

Hematological measures showed significantly increased leukocytes in e-cig or dual users compared with never smokers, driven by an increase in granulocytes, although the specific type(s) of granulocyte being affected—neutrophils, eosinophils, or basophils—is unknown. It is likely, however, that this increase is at least in part due to increased neutrophils, an increase that has been associated with the use of cigarettes (343-347), as neutrophils serve as mediators of inflammation (348-350). Although leukocytes were increased in cigarette only users, this increase was not significant compared with never

smokers. Several epidemiological studies have found increased levels of leukocytes in cigarette smokers (345, 351-355), a change likely indicative of the persistent inflammation induced by MCS (345, 354, 356-358), and while the effects of e-cigs on hematological measures are still not well defined, a small study of cigarette smokers and never-smokers compared CBC markers after cigarette and e-cig use and found that although leukocytes, lymphocytes, and granulocytes were increased after cigarette smoking, they were unchanged after e-cig use (359). It is therefore likely that the increase in leukocytes seen in our e-cig or dual use group is mostly contributable to increased levels of leukocytes in individuals using both traditional and emerging tobacco products.

Measures by CBC also showed increased hemoglobin in e-cig or dual users. Although some prior studies have shown increases in hemoglobin in smokers (339, 349, 354), these changes were not seen in the current study. As previously mentioned, there are few studies of the hematological impacts of e-cig use, but Okuni-Watanabe et al. (360) report a case study showing that hemoglobin, as well as hematocrit, was initially increased in an e-cig user but decreased upon the cessation of e-cig use. Although this case report was based on a single individual—an individual who was also a former heavy smoker—it does suggest the potential for e-cig-related changes in erythrocytic measures. Additional studies are needed to better clarify the effects of e-cig use on hematological measures in both e-cig only users and in dual users.

Red blood cell distribution width (RDW) was found to be significantly higher in cigarette only users compared with both never smokers and e-cig or dual users. Elevated RDW has been previously seen in smokers (361-363) and has been associated with increased morbidity and mortality (362, 364-367). Although the mechanism between

RDW and subsequent morbidity and mortality has not been clarified, this finding in our study may be predictive of future cardiovascular events in the cigarette-smoking participants. Contrastingly, platelet mononuclear cell aggregates (PMAs), a marker of increased thrombosis, were found to be significantly decreased in e-cig or dual users compared with all other groups. Smoking has been long been known to increase platelet aggregation (368), as well as platelet leukocyte aggregation (369), and studies of e-cig exposure have shown similar outcomes (370-372). However, although our e-cig or dual user participants showed no significant difference in platelet count (as measured by CBC) compared with any other use group, they showed decreased PMAs. The reason for this decrease is unknown, and future studies of cigarette only, e-cig only, and dual users should continue to measure PMAs and measure additional markers of thrombosis to better understand this finding.

Unsurprisingly, most urinary VOC metabolites showed higher levels in current tobacco product users compared with never and/or former smokers. Cigarettes are a known source of VOCs (160) as well as one of the leading sources of nicotine (373), and both cigarette only users and e-cig or dual users also showed increased levels of tobacco alkaloids compared with both never and former smokers. Concentrations of nicotine can vary in e-cigs, as different flavorings can contain different levels of nicotine (374), as can levels of VOCs in relation to varying power outputs (96, 375), making measurements of these compounds in e-cig aerosols difficult. However, several studies have shown that, as in the current study, levels of VOCs generated by e-cig devices are lower than levels generated by MCS but still increased compared with levels in nonsmokers (180, 207, 211, 375). Our study supports these findings, as several urinary VOC metabolites were

significantly lower in e-cig or dual users compared with cigarette only smokers, including both metabolites of acrolein (CEMA and 3HPMA) and the metabolite of crotonaldehyde (HPMMA), despite similar levels of urinary cotinine. Although these data support the hypothesis that e-cigs may be less harmful than traditional cigarettes, they also contribute to the growing body of evidence against the claims that e-cigs are harmless (227, 376-378). More studies are needed to better clarify levels of VOCs and tobacco alkaloids generated by e-cigs compared with traditional cigarettes and to determine whether or not any differences in levels between the two types of products indicate a decreased risk of tobacco product-related CVD.

CACs were also examined in relation to tobacco product use categories. Previous research has shown associations between MCS and CACs (72, 316, 318, 320) as well as between acrolein, one of the major constituents of tobacco smoke, and CACs (71, 74, 151). In our models, age and SES were identified as variables affecting levels of CACs independent of tobacco product use categories. Age is a known risk factor for CVD and is included as a variable in a number of CVD risk score calculations (379-382). Age is also associated with decreased endothelium function (383-385), likely through decreased levels and function of CACs (385, 386). Likewise, SES has been shown to have a significant impact on CVD health (387), with this impact thought to be the result of factors such as poor diet (388), decreased access to health care (389), increased presence of roadways (294), and decreased levels of greenness (295) or some combination of these factors and/or others. Both greenness (295) and presence of roadways (294) have previously been associated with changes in the levels of CACs.

Of the fifteen CAC populations measured in our cohort, only two showed

significant associations with any of the tobacco product use categories compared with never smokers after adjustment for age and SES: CAC-12 (45+) and CAC-3 (31+/34+/133+/45_{dim}). CAC-12 is a circulating hematopoietic cell which expresses the leukocyte antigen CD45. In our cohort, e-cig or dual users had significantly lower levels of these cells than never smokers. Although exposure to MCS is associated with decreased levels of CACs, there are still gaps in our understanding of the effect of e-cigs on CACs, particularly populations such as CAC-12, which is a hematopoietic cell but not a hematopoietic stem cell (CD45+/CD34+/CD31+) or an endothelial progenitor cell (CD45_{dim}/CD34+/CD31+). In a previous study, Yeager et al. (295) found that CAC-12, among many of the measured CAC populations, was inversely associated with greenness while other CAC populations derived from CAC-12 (CAC-14 and CAC-4) were positively associated. The presence of the CD45 antigen appeared to contribute to the reparative capacity of the cells positively associated with nearby greenness, as cells displaying the same endothelial and stem cell markers (CD31+/CD34+/133+) but not displaying the CD45 antigen (CD45- or CD45_{dim}) did not show the same response (295). Additional research is needed in order to better understand the true significance of the CD45 antigen in CACs and its potential contribution to the regenerative and reparative capacities of hematopoietic stem cells.

Conversely, the results of our study showed an increase in CAC-3 in e-cig or dual use compared with no product use, past or present. Although smoking has been found to decrease level of CACs, it may be that, as seen with higher levels of roadway proximity (294), the increased levels of CAC-3 measured in e-cig or dual users are indicative of a low yet persistent level of vascular injury that necessitates increased levels of this early

progenitor cell population for repair of the endothelium. This insult may be mild enough to induce increased production of CAC-3 without affecting cell mobilization from the bone marrow, an outcome noted in response to exposure to PM_{2.5} (67) and acrolein (74). Regardless, as levels of the CAC populations that give rise to CAC-3 were not significantly affected by the use of cigarettes and/or e-cigs, the changes noted in CAC-3 may indicate that this population is particularly sensitive to some component to which e-cig or dual users are exposed.

Interestingly, a study of CACs in type 2 diabetics showed that of the CAC populations measured, only CAC-3 was associated with peripheral endothelium function as measured by reactive hyperemia index (RHI), with individuals with higher levels of CAC-3 showing better endothelium function (390). CAC-3 was thus considered the CAC most reflective of endothelium health and was suggested as a specifically sensitive predictor of CVD risk (390). These data also seem to support the hypothesis that the increased levels of CAC-3 in e-cig or dual users compared with never smokers is reflective of increased endothelium function as the vasculature works to repair low levels of injury. Similar increases in CACs are noted in response to acute exposure to e-cig aerosol (322) as well as to MCS (315) and PM (73, 391), while chronic exposures to MCS or PM cause decreases in CACs as endothelium damage overwhelms repair capabilities. As e-cig use is still a fairly acute phenomenon—that is, the majority of users have not been using e-cigs long enough to develop any potential adverse health outcomes that may be associated with chronic use—the increased levels of CAC-3 noted in our study may be reflective of the increase in CACs related to acute exposure, while the increase in dual users may be reflective of the increases in CACs noted with lighter cigarette use (318). Additional

studies of more chronic e-cig and dual use would help clarify whether this increase persists or whether, as with MCS and PM, the levels of CAC-3 decrease.

Given that both these cell populations were only associated with the e-cig or dual use group, we cannot definitively say whether these changes were induced by sole e-cig use or by the combined use of cigarettes and e-cigs. However, as no significant differences were seen in the level of these CACs between cigarette only smokers and never smokers, it is likely that the changes seen in CAC-3 and CAC-12 are related more so to the use of e-cigs. Additional studies with a larger population of e-cig users are needed to better understand these relationships, particularly the relationship with CAC-3, which may specifically have the potential to serve as a biomarker for endothelial function and disease development as previously suggested (390).

The Louisville Healthy Heart Study represents a cross-sectional study examining associations between the use of tobacco products and fifteen antigenetically distinct CAC populations. Although our small number of e-cig only users required their inclusion in an 'e-cig or dual use' group and thus somewhat limited our capability of thoroughly assessing the effect of these devices on CACs as well as on urinary VOC and tobacco alkaloid metabolites and hematological measures, a number of significant differences were noted between this group and the group of individuals smoking cigarettes alone. These differences suggest that e-cig use may produce harmful effects in these outcomes that are distinct from those seen in response to use of cigarettes in addition to the outcomes showing similar responses, such as increased leukocytes. Furthermore, as most e-cig users are actually dual users (392, 393), the results of our combined e-cig or dual use group may be more reflective of real-world exposure to e-cigs and subsequent health outcomes. Our e-

cig or dual user group was significantly younger than other use groups, supporting national studies reporting increased use of e-cigs among younger generations (182, 183, 394), and reported less prevalence of CVD, likely related to their age. Because a number of participants were recruited from CVD clinics at nearby hospitals, the prevalence of CVD and use of CVD medications were unsurprisingly similar between never smokers, former smokers, and cigarette smokers.

Also unsurprising were the significantly higher levels of urinary VOC metabolites in both groups of current tobacco product use compared with never and former smokers, as tobacco products are known to be a significant source of VOCs (160). However, comparison of urinary VOC metabolite levels between cigarette only smokers and e-cig or dual users showed that the use of cigarettes alone resulted in significantly higher levels of several metabolites (CEMA, 3HPMA, AAMA, 3MHBMA, HPMMA, AMCC, and ANTB). Nonetheless, the levels of these metabolites were still significantly higher in e-cig or dual users compared with never or former smokers, so a decreased but continued use of traditional cigarettes in combination with the use of e-cigs will likely still result in levels of VOC exposure that are capable of contributing to CVD development. Likewise, because e-cigs are often considered to be safer than traditional cigarettes (173, 175, 181), e-cig only users may not be aware of the high levels of VOCs to which they are being exposed and the associated health risks.

Analysis of CACs revealed that two cell populations were significantly different between e-cig or dual users and never smokers. CAC-3, an early endothelial progenitor cell population, was significantly increased in e-cig or dual users, while CAC-12, a hematopoietic cell, was significantly decreased. The increased levels of CAC-3 are likely

reflective of a repair mechanism in response to a low but persistent level of injury in the endothelium. The cause in the decrease in levels of CAC-12, despite being strongly associated with e-cig or dual use as evidenced by its significance in all tested models, is less clear and will require additional, more specific studies to better understand. That both these differences were significant despite a small sample in our e-cig or dual use group speaks to their importance and potentially to their relevance in other CVD effects induced by the use of e-cigs alone or in combination with traditional cigarettes.

With the increasing use of e-cigs and other ENDS, it is essential to understand the health risks associated with their use. Although few studies have examined the risks of e-cigs on cardiovascular health, the changes noted in hematological measures and in CACs in the LHHS and in other human cohorts (322) demonstrate the potential for these devices to contribute to increased CVD risk, as does the evidence of high levels of VOCs in e-cig aerosol both in our study and in others (395). Additional research is needed to clarify the results of these early e-cig studies and to explore the specific findings shown in the current study. Furthermore, more studies focusing on e-cig and dual users would help elucidate how the combined use of e-cigs and traditional cigarettes may change CVD risk, that is, whether combined use is healthier or safer than use of cigarettes alone. A better understanding of the impacts of e-cigs on overall health would help medical professionals develop better methods of care for both acute and chronic adverse effects resulting from the use of these products.

CHAPTER IV

SYSTEMIC, HEMATOLOGICAL, AND ENDOTHELIUM-RELATED EFFECTS OF MAINSTREAM CIGARETTE SMOKE, E-CIGARETTE AEROSOL, AND PROPYLENE GLYCOL:VEGETABLE GLYCERIN IN MICE

A. Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide (1, 2). In 2016, more than one-fourth of the almost 57 million global deaths were attributed to CVD, and ischemic heart disease and stroke have been classified as the two leading causes of death for the last 20 years (2). In the United States alone, on average, an individual dies of CVD every 38 seconds; this means that more than 2,000 individuals die every day from some form of CVD (1). Although a number of physical, behavioral, and socioeconomic factors have been linked to CVD (3), tobacco smoke has been characterized as the most significant modifiable risk factors in CVD development (14, 75). Tobacco smoke contains thousands of compounds, including up to 100,000 unidentified compounds (396), and many of these can contribute to disease development even in very small quantities (82, 396). Extensive evidence demonstrates that exposure to both mainstream (MCS) (80, 82) and secondhand (SHS) (80, 84) cigarette smoke increase the risk for CVD. Smoking has been shown to increase the risk of heart attack, coronary artery disease, atherosclerosis, and stroke (85). Smoking has also been linked to endothelium

dysfunction (85), a *sine qua non* for CVD. However, the pathophysiological mechanisms through which tobacco smoke mediates and promotes CVD are not well understood.

Although American smoking rates are decreasing (1, 119), the number of individuals using electronic cigarettes (e-cigs) is increasing, especially among youths and never smokers (1, 182, 183, 325, 397), and although e-cigs are promoted as being healthier than traditional cigarettes (173-175, 177, 184), their true health effects are not yet known. Early studies of e-cigs have shown that the generated aerosol can contain many harmful or potentially harmful components, including particulates (184, 198-202), metals (184, 203-205), and volatile organic compounds (VOCs) including aldehydes (96, 180, 199, 201, 206-213). The potential health effects of the liquid components are also causing growing concern, as humectants including propylene glycol (PG) and vegetable glycerin (VG) have been linked to aldehyde generation (168, 214-216) and as many of the flavoring additives used to make flavored e-cig liquids have been deemed safe for ingestion but not necessarily for inhalation (218). As the morbidity and mortality from e-cigarette, or vaping, product use associated lung injury (EVALI) has increased in the United States over the past year (225-229), it has become imperative to study the health effects of e-cigs and their various components. Thus, the goal of these experiments was to determine the effects of MCS, e-cig aerosol, and PG:VG on CACs as well as systemic and hematological biomarkers.

B. Experimental Procedures

1. Exposures

Animals were exposed to mixtures or humectants via full-body inhalation as described in Chapter II, Sections B.2. Upon completion of the exposure, mice were euthanized and tissues collected as described in Chapter II, Section B.5.

2. Urine Collection

Urine samples were collected post-exposure as described in Chapter II, Section B.3.

3. Urine Metabolite Quantification

Collected urine was used to quantify levels of VOC and tobacco alkaloid metabolites using analytical methods as described in Chapter II, Section B.4.

4. Complete Blood Cell Count (CBC)

A sample of whole blood was collected after the final day of exposure for a complete blood cell count (CBC) as described in Chapter II, Section B.6.

5. Plasma Biomarkers Quantification

Plasma was prepared from each sample for an analysis of a number of biomarkers as described in Chapter II, Section B.7.

6. Circulating Angiogenic Cell (CAC) Quantification

Flow cytometry was used to analyze the number of circulating angiogenic cells (CACs) in each sample as described in Chapter II, Section B.8.

7. Statistical Analyses

Statistical analyses were performed as described in Chapter II, Section B.10.

C. Results

1. Urinary Metabolites in MCS-Exposed Mice

The use of tobacco products is the leading cause of preventable death both nationally and worldwide (1). Mainstream cigarette smoke (MCS) has been shown to contain thousands of compounds (396), and many of these have been implicated in disease development (80-82, 396). To better understand how specific components of MCS—mainly, aldehydes—may be contributing to disease development, specifically CVD, we

measured urinary formate, acetate, 3-hydroxypropylmercapturic acid (3HPMA), and 3-hydroxy-1-methylpropylmercapturic acid (HPMMA), as well as total urinary nicotine metabolites (nicotine, cotinine, and trans-3'-hydroxycotinine (3HC)) by gas chromatography/mass spectrometry (GC-MS) or ultra-performance liquid-chromatography tandem mass spectrometry (UPLC-MS/MS) as biomarkers of MCS-related aldehyde exposure. For this, male mice were exposed to MCS (50% of smoke of 12 KY Reference 3R4F cigarettes, 6h), and urine was collected immediately after this single exposure session. Urinary levels of formate ($p=0.01$), 3HPMA ($p=0.01$), HPMMA ($p<0.001$), and total nicotine metabolites ($p<0.001$) measured within the first 3 hours post-exposure significantly exceeded levels measured after a 6h exposure to HEPA (Fig. 4.1). Levels of formate and acetate after the overnight (3-16h) collection were elevated compared with levels 0-3h post-exposure; these elevations are related to generation by food, as mice were given free access to food during this collection period (Fig. 4.1).

2. Systemic Toxicity of Acute Exposure to Inhaled MCS

Tobacco use is responsible for an estimated 7 million global deaths each year (398), with many of these deaths due to CVD (399). Tobacco smoke contains thousands of compounds, including many unidentified compounds, many of which can contribute to CVD development even in very small quantities (82, 396). We therefore tested for changes in systemic and hematological biomarkers in mice exposed to MCS (50% of smoke of 12 KY Reference 3R4F cigarettes). Acute (6h/day; 4d) exposure had modest effects on organ and body weights (or ratios) and CBCs. MCS exposure caused a lower final body weight (-9%; $p=0.003$), liver/body weight ratio (-17%; $p=0.02$), and, modestly, heart/body weight ratio (-8%; $p=0.06$) (Table 4.1) than exposure to air. The overall leukocyte count was

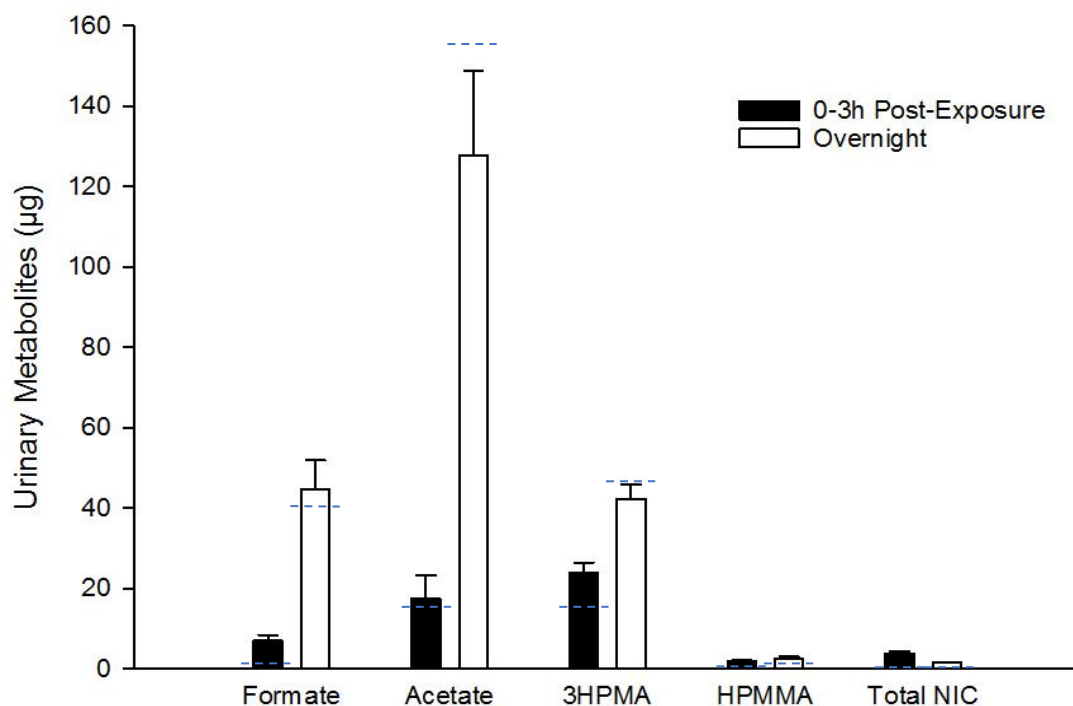


Fig. 4.1. Urinary metabolites after acute mainstream cigarette smoke (MCS) exposure. Male mice were exposed (6h) to mainstream cigarette smoke (MCS; 50% of smoke of 12 KY Reference 3R4F cigarettes) after which urine was collected at 0–1, 1–2, 2–3, and 3–16h (overnight) and aldehyde and tobacco alkaloid metabolites quantified. Values are mean \pm SE (n=12 mice per group). Dashed lines indicate metabolite measures (μ g) after HEPA exposure (6h). **Abbr.:** **3HPMA**, 3-hydroxypropylmercapturic acid; **HPMMA**, 3-hydroxy-1-methylpropylmercapturic acid; **Total NIC**, total urinary nicotine metabolites (nicotine, cotinine, and trans-3'-hydroxycotinine).

Table 4.1. Systemic parameters of male C57BL/6 mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 12 KY Reference 3R4F cigarettes).

| Variable | Exposure (4 days) | |
|------------------------------|-------------------|----------------|
| | Air Control | MCS |
| Final BWT (g) | 30 ± 0 | 27 ± 0* |
| Heart/BWT (mg/g) | 5.5 ± 0.2 | 5.0 ± 0.1# |
| Lung/BWT (mg/g) | 5.9 ± 0.2 | 5.7 ± 0.3 |
| Liver/BWT (mg/g) | 48.4 ± 2.6 | 40.4 ± 0.7* |
| Kidney/BWT (mg/g) | 13.9 ± 0.4 | 13.3 ± 0.1 |
| Plasma Measurements | | |
| Cholesterol (mg/dL) | 102.97 ± 2.56 | 100.25 ± 3.72 |
| Triglycerides (mg/dL) | 28.75 ± 6.19 | 25.91 ± 2.11 |
| Albumin (g/dL) | 3.53 ± 0.17 | 3.96 ± 0.04* |
| Total Protein (g/dL) | 4.88 ± 0.19 | 4.94 ± 0.05 |
| ALT (U/I) | 21.55 ± 1.19 | 22.63 ± 0.65 |
| AST (U/I) | 52.67 ± 5.41 | 62.93 ± 6.89 |
| CK (U/I) | 251.56 ± 45.78 | 234.16 ± 31.19 |
| LDH (U/I) | 185.12 ± 5.76 | 187.65 ± 9.32 |
| Creatinine (mg/dL) | 0.49 ± 0.10 | 0.35 ± 0.01 |

Values = mean ± SE (n=4-7 mice per group); Creatinine compared using Rank Sum Test. Abbr.: **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **BWT**, body weight; **CK**, creatine kinase; **LDH**, lactate dehydrogenase; **MCS**, mainstream cigarette smoke; *, p<0.05 compared with air control based on Bonferroni's or Dunn's post-test; #, 0.05≤p≤0.10 compared with air control based on Bonferroni's or Dunn's post-test.

significantly decreased with exposure (-60%; $p=0.01$), driven by a decrease in lymphocytes (-58%; $p=0.01$), as was mean corpuscular volume (-10%; $p=0.002$), while mean corpuscular hemoglobin concentration was increased (+11%; $p<0.001$) (Table 4.2). Plasma levels of liver (ALT, AST), muscle (CK), and LDH enzymes and creatinine were unaffected by exposure, but albumin was significantly increased (+12%; $p=0.004$) (Table 4.1).

3. Acute Exposure to MCS and Circulating Angiogenic Cells

Acute (4d) exposure to MCS (50% of smoke of 12 KY Reference 3R4F cigarettes) caused a significant decrease in levels of Sca-1⁺ cells (-56%; $p=0.03$) (Fig. 4.2A) but did not change the levels of CACs (Fig. 4.2B).

4. Urinary Metabolites in JUUL E-Cig-Exposed Mice

The use of e-cig devices has been increasing dramatically in both adolescents and adults over the past several years (83, 181-183, 325). Although e-cigs are commonly touted as a healthier alternative to traditional cigarettes (173-175, 177, 181, 184), the health effects of these devices are still largely unknown. Furthermore, a number of studies have detected harmful constituents including aldehydes in the aerosol of these devices, which may indicate that e-cigs can contribute to adverse health risks in a fashion similar to traditional cigarettes (96, 180, 207, 211). To better characterize exposure to e-cig aerosol, we measured urinary formate, acetate, 3HPMA, and total urinary nicotine metabolites (nicotine, cotinine, and 3HC) by GC-MS and UPLC-MS/MS to evaluate the presence of aldehydes in aerosols generated by e-cig devices. For this, male mice were exposed to aerosol from flavored JUUL e-cig devices (Virginia (VA) Tobacco, Mango, or Menthol;

Table 4.2. Hematological measures of male C57BL/6 mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 12 KY Reference 3R4F cigarettes).

| Hematological Measurements | Exposure (4 days) | |
|--|-------------------|--------------|
| | Air Control | MCS |
| White Blood Cell (K/uL) | 1.69 ± 0.21 | 0.68 ± 0.09* |
| Neutrophils (K/uL) | 0.47 ± 0.09 | 0.15 ± 0.02 |
| Lymphocytes (K/uL) | 1.17 ± 0.17 | 0.50 ± 0.07* |
| Monocytes (K/uL) | 0.05 ± 0.01 | 0.03 ± 0.01 |
| Eosinophils (K/uL) | 0 ± 0 | 0 ± 0 |
| Basophils (K/uL) | 0 ± 0 | 0 ± 0 |
| Red Blood Cell (M/uL) | 8.31 ± 0.14 | 8.78 ± 0.14 |
| Hemoglobin (g/dL) | 11.6 ± 0.5 | 12.1 ± 0.1 |
| Hematocrit (%) | 39.0 ± 1.8 | 36.8 ± 0.6 |
| Mean Corpuscular Volume (fL) | 46.8 ± 1.4 | 41.9 ± 0.6* |
| Mean Corpuscular Hemoglobin (pg) | 13.9 ± 0.4 | 13.8 ± 0.1 |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 29.7 ± 0.1 | 32.9 ± 0.3* |
| Red Cell Distribution Width (%) | 17.6 ± 0.2 | 17.9 ± 0.2 |
| Platelets (K/uL) | 912 ± 105 | 1049 ± 28 |
| Mean Platelet Volume (fL) | 4.6 ± 0.1 | 4.5 ± 0 |

Values = mean ± SE (n=4-5 mice per group); Abbr.: **MCS**, mainstream cigarette smoke; *, p<0.05 compared with air control based on Bonferroni's or Dunn's post-test.

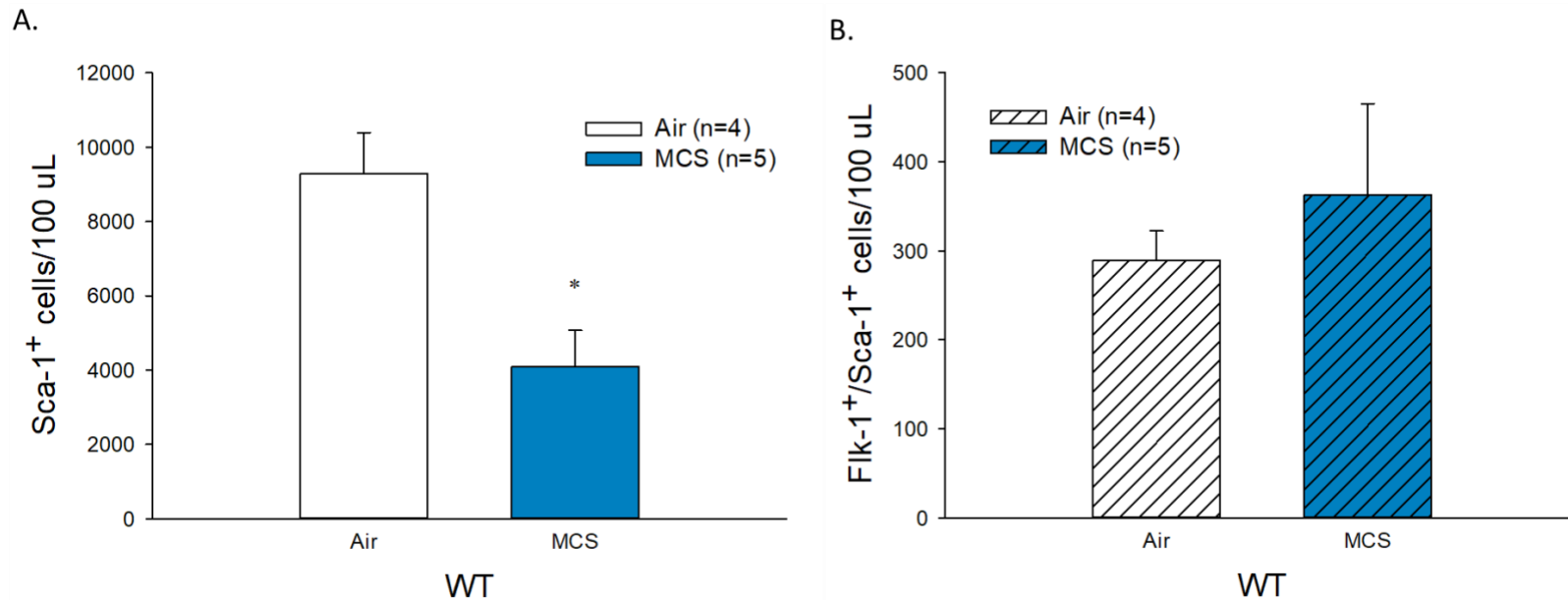


Fig. 4.2. Effects of acute mainstream cigarette smoke (MCS) exposure on circulating stem cells in male mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in male C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or MCS (50% of smoke of 12 KY Reference 3R4F Cigarettes, 6h/d) for 4 days. After 4 days of exposure to MCS, the level of circulating Sca-1⁺ cells was significantly decreased in WT mice (**A**), however, no change in the level of CACs (Flk-1⁺/Sca-1⁺ cells) was detected relative to air-exposed control mice (**B**). Values = mean \pm SE (n=4-5 mice per group). *, p<0.05 compared with air control based on Bonferroni's or Dunn's post-test.

6h), and urine was collected immediately after the exposure sessions. Although acrolein has been measured in e-cig aerosol, urinary levels of 3HPMA after the JUUL exposures were not significantly different from levels after acute air exposure (6h) (Figs. 4.3, 4.4, & 4.5). Likewise, levels of urinary formate and acetate were not significantly different from levels in air-exposed mice (Figs. 4.3, 4.4, & 4.5). Measures of total urinary nicotine metabolites, however, were significantly elevated post-JUUL exposure (VA Tobacco: $p < 0.001$; Mango and Menthol: $p = 0.002$); mice exposed to either JUUL Mango or JUUL Menthol showed similar levels of urinary nicotine ($p = 0.2$), which were significantly higher than levels in mice exposed to JUUL VA Tobacco ($p = 0.02$ for both) (Figs. 4.3, 4.4, & 4.5). Levels of formate and acetate after the overnight (3-16h) collection were elevated compared with levels 0-3h post-exposure for exposure to all three JUUL products; these elevations are likely related to generation by food, as mice were given free access to food during this collection period (Figs. 4.3, 4.4, & 4.5).

5. Systemic Toxicity of Acute Exposure to Inhaled JUUL Mango E-cig Aerosol

Because of the known health effects of aldehydes found to be generated in the aerosol of e-cigarette (e-cig) devices (96, 180, 207-213) and because of the potential risks of flavorings used in these devices (183, 218, 220-223), we tested for changes in systemic and hematological biomarkers in female mice exposed to aerosol from a JUUL e-cig device with mango flavoring (JUUL Mango). Acute (6h/day; 4d) exposure had a number of significant effects on organ and body weights (or ratios) and CBC. For example, exposure to JUUL Mango caused a greater change in body weight compared with air-exposed control mice (-3 g vs -2 g; $p = 0.01$), with a modest increase in heart/body weight ratio (+6%;

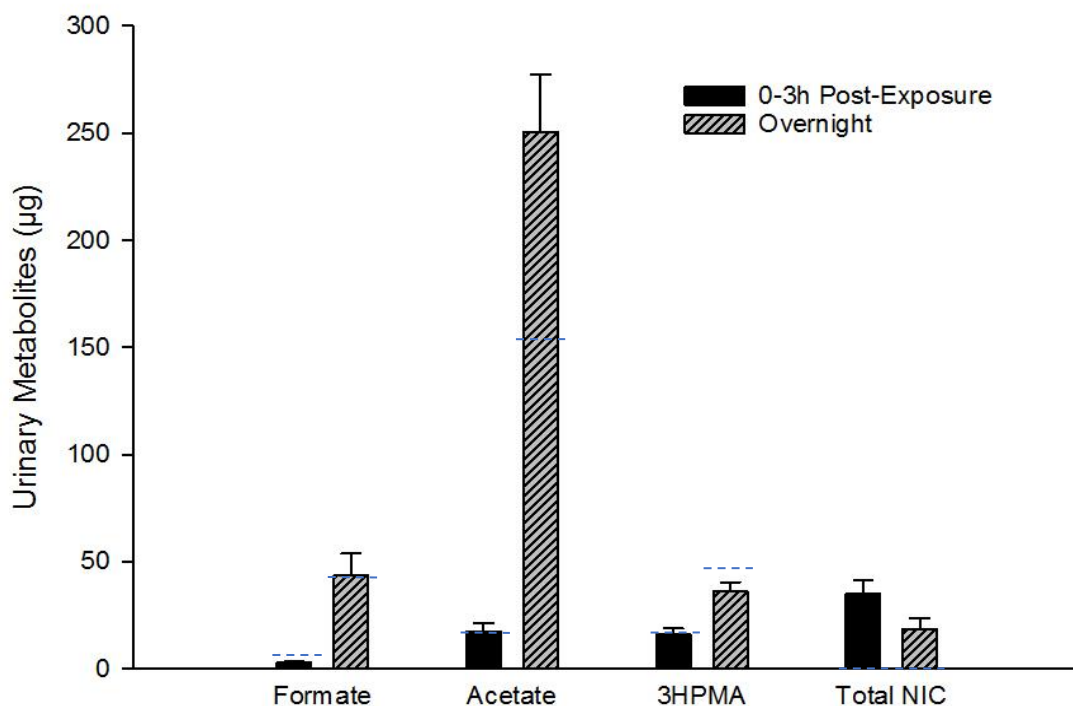


Fig. 4.3. Urinary metabolites after acute electronic cigarette aerosol (e-cig; JUUL VA Tobacco) exposure. Male mice were exposed (6h) to e-cig aerosol (JUUL VA Tobacco) after which urine was collected at 0–1, 1–2, 2–3, and 3–16h (overnight) and aldehyde and tobacco alkaloid metabolites quantified. Values are mean \pm SE (n=5 mice per group). Dashed lines indicate metabolite measures (μg) after HEPA exposure (6h). **Abbr.:** **3HPMA**, 3-hydroxypropylmercapturic acid; **Total NIC**, total urinary nicotine metabolites (nicotine, cotinine, and trans-3'-hydroxycotinine).

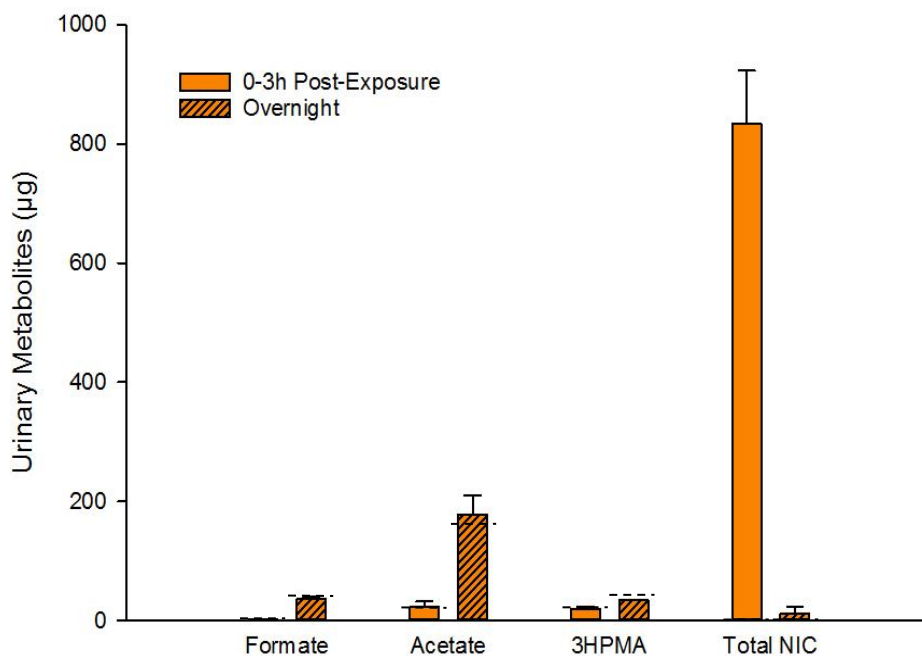


Fig. 4.4. Urinary metabolites after acute electronic cigarette aerosol (e-cig; JUUL Mango) exposure. Male mice were exposed (6h) to e-cig aerosol (JUUL VA Mango) after which urine was collected at 0–1, 1–2, 2–3, and 3–16h (overnight) and aldehyde and tobacco alkaloid metabolites quantified. Values are mean \pm SE (n=4 mice per group). Dashed lines indicate metabolite measures (μg) after HEPA exposure (6h). **Abbr.:** **3HPMA**, 3-hydroxypropylmercapturic acid; **Total NIC**, total urinary nicotine metabolites (nicotine, cotinine, and trans-3'-hydroxycotinine).

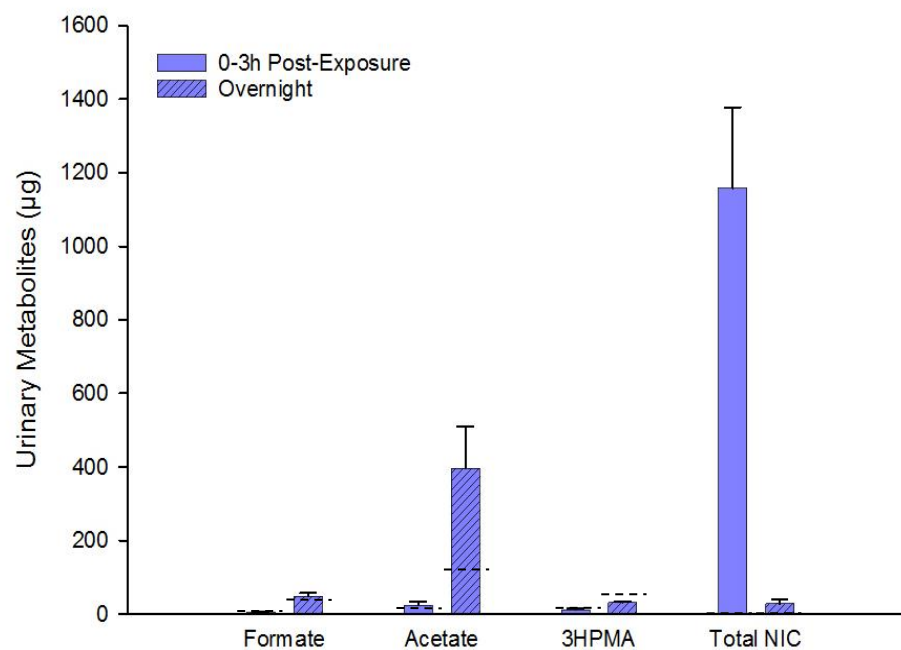


Fig. 4.5. Urinary metabolites after acute electronic cigarette aerosol (e-cig; JUUL Menthol) exposure. Male mice were exposed (6h) to e-cig aerosol (JUUL Menthol) after which urine was collected at 0–1, 1–2, 2–3, and 3–16h (overnight) and aldehyde and tobacco alkaloid metabolites quantified. Values are mean \pm SE (n=4 mice per group). Dashed lines indicate metabolite measures (μg) after HEPA exposure (6h). **Abbr.:** **3HPMA**, 3-hydroxypropylmercapturic acid; **Total NIC**, total urinary nicotine metabolites (nicotine, cotinine, and trans-3'-hydroxycotinine).

p=0.06) and a significant increase in kidney/body weight ratio (+7%; p=0.045) (Table 4.3). The overall leukocyte count was significantly decreased (-48%; p=0.001), driven by decreases in neutrophils (-54%; p=0.004), lymphocytes (-46%; p=0.002), and monocytes (-48%; p=0.06) (Table 4.4). However, changes in erythrocytic measures were only seen in hemoglobin (+3%; p=0.02) and hematocrit (+4%; p=0.05) (Table 4.4). Although plasma levels of muscle (CK) and LDH enzyme and creatinine were unchanged in exposed mice, exposure caused modestly significant increases in both albumin (+8%; p=0.06) and AST (+34%; p=0.06) (Table 4.3).

6. Acute JUUL Mango E-cig Aerosol Exposure and CACs

Acute (4d) exposure to JUUL Mango aerosol caused modest decreases in levels of both Sca-1+ cells (-67%; p=0.06) and CACs (-75%; p=0.06) (Fig. 4.6A & B).

7. Systemic Toxicity of Acute Exposure to blu Plus+ E-cig Aerosol

E-cig aerosol has been linked to negative health effects (174, 190-192), but the mechanism of these effects has not been identified. Thus, we tested for changes in systemic and hematological biomarkers in mice exposed to e-cig (blu Plus+) aerosol (4d). Acutely-exposed male mice showed a significantly decreased final body weight (-6%; p=0.003) but no changes in measured organ/body weight ratios (Table 4.5). Although plasma levels of liver (ALT, AST), muscle (CK), and LDH enzymes and creatinine were unaffected by exposure, triglycerides were modestly decreased (-12%; p=0.09) (Table 4.5). Measures of toxicity by CBC showed considerable effects. For example, e-cig exposure significantly decreased leukocyte count (-36%; p<0.001), driven by decreases in all leukocyte subpopulations (Table 4.6). Increases in mean corpuscular hemoglobin (+7%; p<0.001), mean corpuscular hemoglobin concentration (+6%; p<0.001), and, modestly, hemoglobin

Table 4.3. Systemic parameters of female C57BL/6 mice acutely exposed to either air or electronic cigarette aerosol (e-cig; JUUL Mango).

| Variable | Exposure (4 days) | |
|------------------------------|-------------------|----------------|
| | Air Control | JUUL Mango |
| Change in BWT (g) | -2 ± 0 | -3 ± 0* |
| Heart/BWT (mg/g) | 5.0 ± 0.1 | 5.3 ± 0.1# |
| Lung/BWT (mg/g) | 6.5 ± 0.2 | 6.6 ± 0.2 |
| Liver/BWT (mg/g) | 42.9 ± 0.7 | 43.5 ± 1.0 |
| Kidney/BWT (mg/g) | 12.7 ± 0.2 | 13.7 ± 0.3* |
| Plasma Measurements | | |
| Cholesterol (mg/dL) | 59.40 ± 1.12 | 56.20 ± 1.77 |
| HDL (mg/dL) | 30.40 ± 0.93 | 29.20 ± 1.24 |
| LDL (mg/dL) | 21.44 ± 0.42 | 20.20 ± 1.10 |
| Triglycerides (mg/dL) | 37.80 ± 0.92 | 34.00 ± 3.61 |
| Albumin (g/dL) | 2.68 ± 0.07 | 2.90 ± 0.05# |
| Total Protein (g/dL) | 4.46 ± 0.09 | 4.66 ± 0.09 |
| ALT (U/I) | 7.20 ± 0.92 | 7.60 ± 0.60 |
| AST (U/I) | 35.75 ± 2.63 | 47.80 ± 4.42# |
| CK (U/I) | 105.25 ± 7.12 | 175.60 ± 47.54 |
| LDH (U/I) | 120.20 ± 19.34 | 117.80 ± 6.15 |
| Creatinine (mg/dL) | 0.19 ± 0.01 | 0.19 ± 0.01 |

Values = mean ± SE (n=5-10 mice per group); Abbr.: **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **BWT**, body weight; **CK**, creatine kinase; **E-cig**, electronic cigarette; **HDL**, high-density lipoprotein; **LDH**, lactate dehydrogenase; **LDL**, low-density lipoprotein; *, p<0.05 compared with air control; #, 0.05≤p≤0.10 compared with air control.

Table 4.4. Hematological measures of female C57BL/6 mice acutely exposed to either air or electronic cigarette aerosol (e-cig; JUUL Mango).

| Hematological Measurements | Exposure (4 days) | |
|--|-------------------|--------------|
| | Air Control | JUUL Mango |
| White Blood Cell (K/uL) | 1.15 ± 0.13 | 0.60 ± 0.07* |
| Neutrophils (K/uL) | 0.23 ± 0.03 | 0.11 ± 0.01* |
| Lymphocytes (K/uL) | 0.88 ± 0.10 | 0.48 ± 0.06* |
| Monocytes (K/uL) | 0.03 ± 0.01 | 0.02 ± 0# |
| Eosinophils (K/uL) | 0 ± 0 | 0 ± 0 |
| Basophils (K/uL) | 0 ± 0 | 0 ± 0 |
| Red Blood Cell (M/uL) | 9.07 ± 0.08 | 9.33 ± 0.14 |
| Hemoglobin (g/dL) | 12.6 ± 0.1 | 13.0 ± 0.1* |
| Hematocrit (%) | 46.9 ± 0.5 | 48.6 ± 0.5# |
| Mean Corpuscular Volume (fL) | 51.7 ± 0.3 | 51.5 ± 0.2 |
| Mean Corpuscular Hemoglobin (pg) | 13.8 ± 0.1 | 13.8 ± 0.1 |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 26.8 ± 0.1 | 26.7 ± 0.2 |
| Red Cell Distribution Width (%) | 16.3 ± 0.1 | 16.2 ± 0.1 |
| Platelets (K/uL) | 723 ± 15 | 694 ± 24 |
| Mean Platelet Volume (fL) | 4.4 ± 0 | 4.3 ± 0 |

Values = mean ± SE (n=10 mice per group); Abbr.: **E-cig**, electronic cigarette; *, p<0.05 compared with air control; #, 0.05≤p≤0.10 compared with air control.

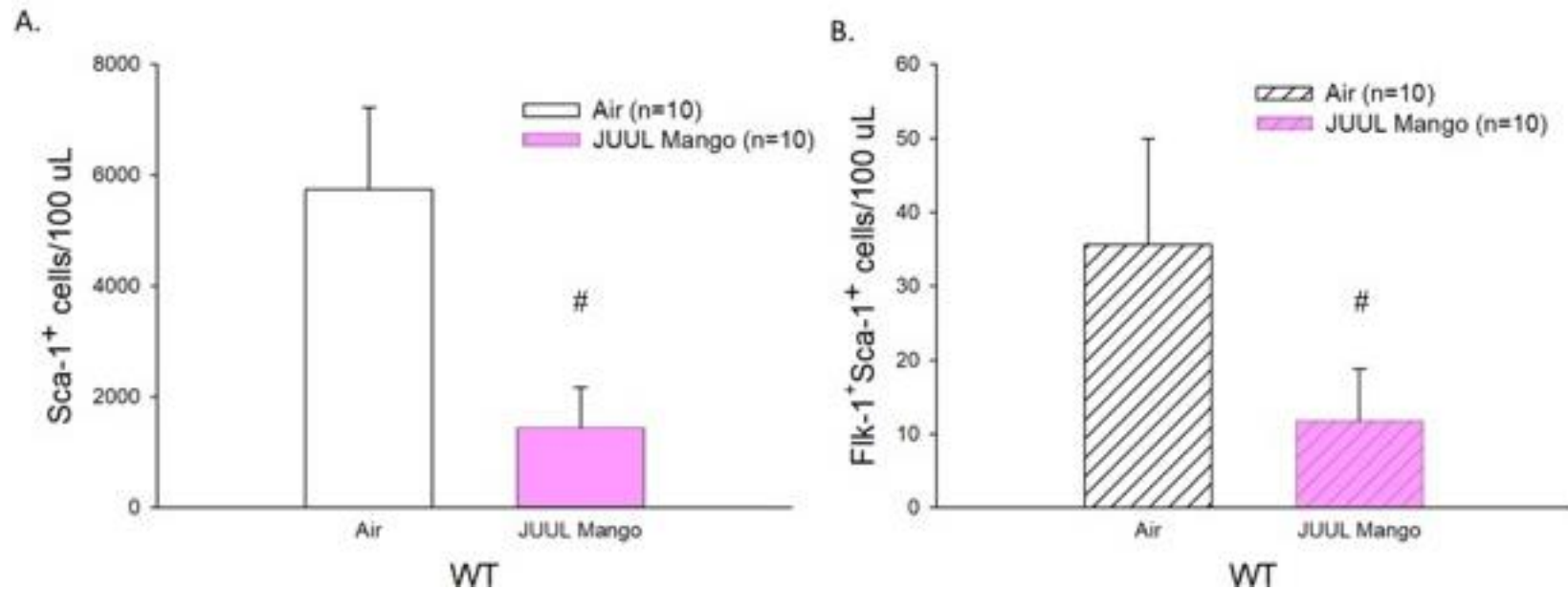


Fig. 4.6. Effects of acute electronic cigarette (e-cig; JUUL Mango) aerosol exposure on circulating stem cells in female mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in female C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or JUUL Mango e-cigarette (e-cig) aerosol (6h/d) for 4 days. After 4 days of exposure to JUUL Mango, the level of both circulating Sca-1⁺ cells (**A**) and the level of CACs (Flk-1⁺/Sca-1⁺ cells) (**B**) decreased relative to air-exposed control mice. Values = mean \pm SE (n=5 mice per group). #, $0.05 \leq p \leq 0.10$ compared with air control.

Table 4.5. Systemic parameters of male C57BL/6 mice acutely exposed to either air or electronic cigarette aerosol (e-cig; blu Plus+).

| Variable | Exposure (4 days) | |
|------------------------------|-------------------|-----------------|
| | Air Control | blu Plus+ e-cig |
| Final BWT (g) | 27 ± 0 | 25 ± 0* |
| Heart/BWT (mg/g) | 5.1 ± 0.1 | 5.2 ± 0.1 |
| Lung/BWT (mg/g) | 5.6 ± 0.1 | 5.8 ± 0.2 |
| Liver/BWT (mg/g) | 45.0 ± 0.8 | 44.3 ± 0.8 |
| Kidney/BWT (mg/g) | 12.1 ± 0.3 | 12.3 ± 0.2 |
| Plasma Measurements | | |
| Cholesterol (mg/dL) | 83.25 ± 2.53 | 78.45 ± 2.78 |
| HDL (mg/dL) | 49.57 ± 1.04 | 44.85 ± 5.50 |
| LDL (mg/dL) | 5.84 ± 0.42 | 6.09 ± 1.07 |
| Triglycerides (mg/dL) | 44.19 ± 2.09 | 39.01 ± 1.69# |
| Albumin (g/dL) | 2.86 ± 0.04 | 2.93 ± 0.05 |
| Total Protein (g/dL) | 4.04 ± 0.16 | 3.89 ± 0.16 |
| ALT (U/I) | 23.46 ± 2.46 | 20.18 ± 2.08 |
| AST (U/I) | 53.03 ± 6.73 | 40.94 ± 2.89 |
| CK (U/I) | 178.42 ± 18.62 | 157.50 ± 11.00 |
| LDH (U/I) | 166.19 ± 12.36 | 155.11 ± 11.36 |
| Creatinine (mg/dL) | 0.23 ± 0.01 | 0.24 ± 0.01 |

Values = mean ± SE (n=9-10 mice per group); Abbr.: **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **BWT**, body weight; **CK**, creatine kinase; **E-cig**, electronic cigarette; **HDL**, high-density lipoprotein; **LDH**, lactate dehydrogenase; **LDL**, low-density lipoprotein; *, p<0.05 compared with air control; #, 0.05≤p≤0.10 compared with air control.

Table 4.6. Hematological measures of male C57BL/6 mice acutely exposed to either air or electronic cigarette aerosol (e-cig; blu Plus+).

| Hematological Measurements | Exposure (4 days) | |
|--|-------------------|-----------------|
| | Air Control | blu Plus+ e-cig |
| White Blood Cell (K/uL) | 1.80 ± 0.16 | 1.15 ± 0.12* |
| Neutrophils (K/uL) | 0.45 ± 0.07 | 0.28 ± 0.05* |
| Lymphocytes (K/uL) | 1.29 ± 0.11 | 0.85 ± 0.08* |
| Monocytes (K/uL) | 0.06 ± 0.01 | 0.03 ± 0* |
| Eosinophils (K/uL) | 0.01 ± 0 | 0 ± 0* |
| Basophils (K/uL) | 0 ± 0 | 0 ± 0 |
| Red Blood Cell (M/uL) | 7.66 ± 0.29 | 7.98 ± 0.33 |
| Hemoglobin (g/dL) | 10.5 ± 0.5 | 11.7 ± 0.6# |
| Hematocrit (%) | 33.6 ± 1.4 | 35.1 ± 1.6 |
| Mean Corpuscular Volume (fL) | 43.7 ± 0.2 | 42.3 ± 1.7 |
| Mean Corpuscular Hemoglobin (pg) | 13.7 ± 0.1 | 14.6 ± 0.4* |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 31.3 ± 0.2 | 33.3 ± 0.4* |
| Red Cell Distribution Width (%) | 16.9 ± 0.2 | 17.4 ± 0.4 |
| Platelets (K/uL) | 592 ± 21 | 4.2 ± 0 |
| Mean Platelet Volume (fL) | 605 ± 30 | 4.4 ± 0.2 |

Values = mean ± SE (n=9-10 mice per group); Abbr.: **E-cig**, electronic cigarette; *, p<0.05 compared with air control; #, 0.05≤p≤0.10 compared with air control.

(+11%; $p=0.10$) were also seen in e-cig-exposed mice compared with air-exposed control mice (Table 4.6).

8. Acute blu Plus+ E-cig Aerosol Exposure and Circulating Angiogenic Cells

Male mice acutely exposed to e-cig (blu Plus+) aerosol (4d) showed no changes in the levels of CACs or Sca-1⁺ cells (Fig. 4.7A & B).

9. Urinary Metabolites in PG:VG-Exposed Mice

PG and VG are commonly used as humectants in e-cig devices (179). The ratio of these compounds can vary between different e-cig devices, as well as with user modification, and variations in these ratios may influence the generation of different compounds in the e-cig aerosol (179). To better characterize exposure to PG:VG in e-cig devices and to determine how varying ratios may impact aldehyde production, we measured urinary formate, acetate, 3HPMA, HPMMA, and/or total urinary nicotine metabolites by GC-MS and UPLC-MS/MS to evaluate the presence of PG:VG-related aldehydes in aerosols generated by e-cig devices. For this, male mice were exposed to PG:VG (50%:50% or 30%:70%, 6h), and urine was collected immediately after exposure. Urinary levels of formate during the first three hours post-exposure were significantly increased in mice exposed to 50%:50% PG:VG compared with control mice ($p=0.003$) (Fig. 4.8), while urinary acetate was increased with exposure to 30%:70% PG:VG ($p=0.047$) (Fig. 4.9). Similarly, the first three hours post-exposure showed significantly increased levels of urinary 3HPMA after exposure to either ratio of PG:VG ($p<0.001$ for 50%:50% PG:VG; $p=0.02$ for 30%:70% PG:VG) (Figs. 4.8 & 4.9). Mice exposed to 50%:50% PG:VG also showed a small but measurable level of HPMMA, the major urinary metabolite of crotonaldehyde, although the measured level was not significantly different

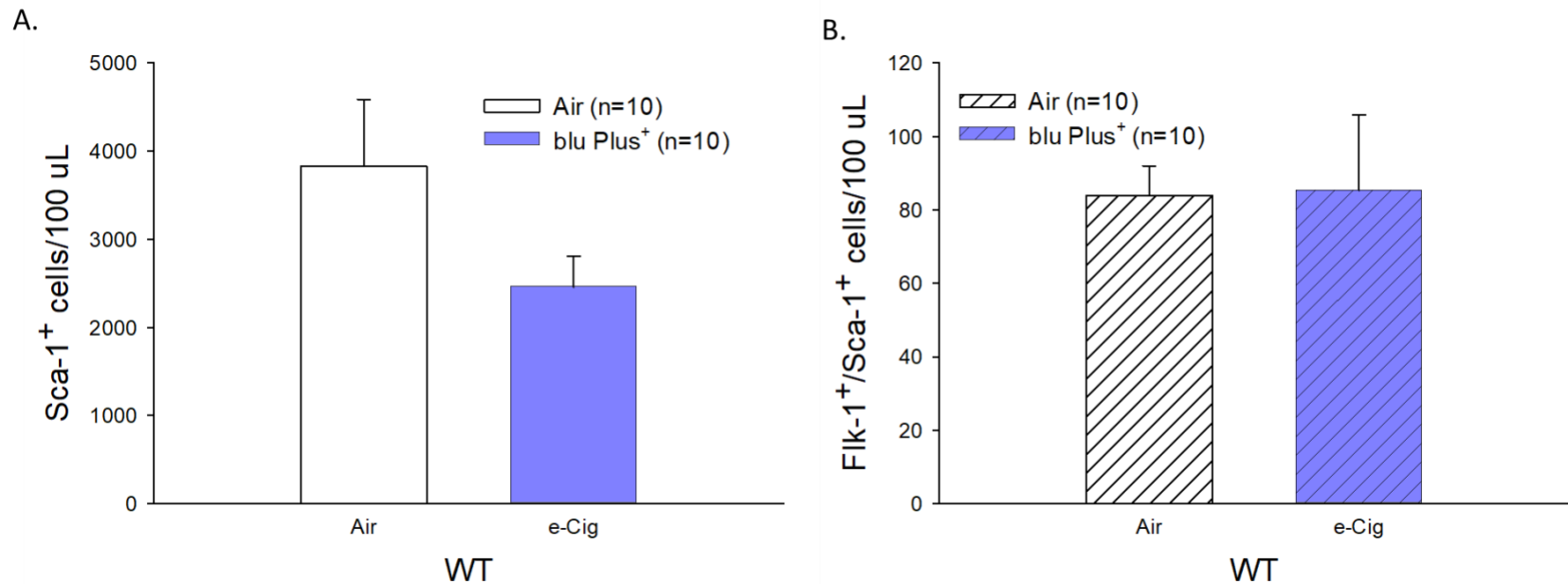


Fig. 4.7. Effects of acute electronic cigarette (e-cig; blu Plus⁺) aerosol exposure on circulating stem cells in male mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in male C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or e-cig aerosol (blu Plus⁺, 6h/d) for 4 days. Levels of circulating Sca-1⁺ cells (**A**) and CACs (Flk-1⁺/Sca-1⁺ cells; **B**) were unchanged in e-cig aerosol-exposed mice relative to air control mice. Values = mean \pm SE (n=10 mice per group).

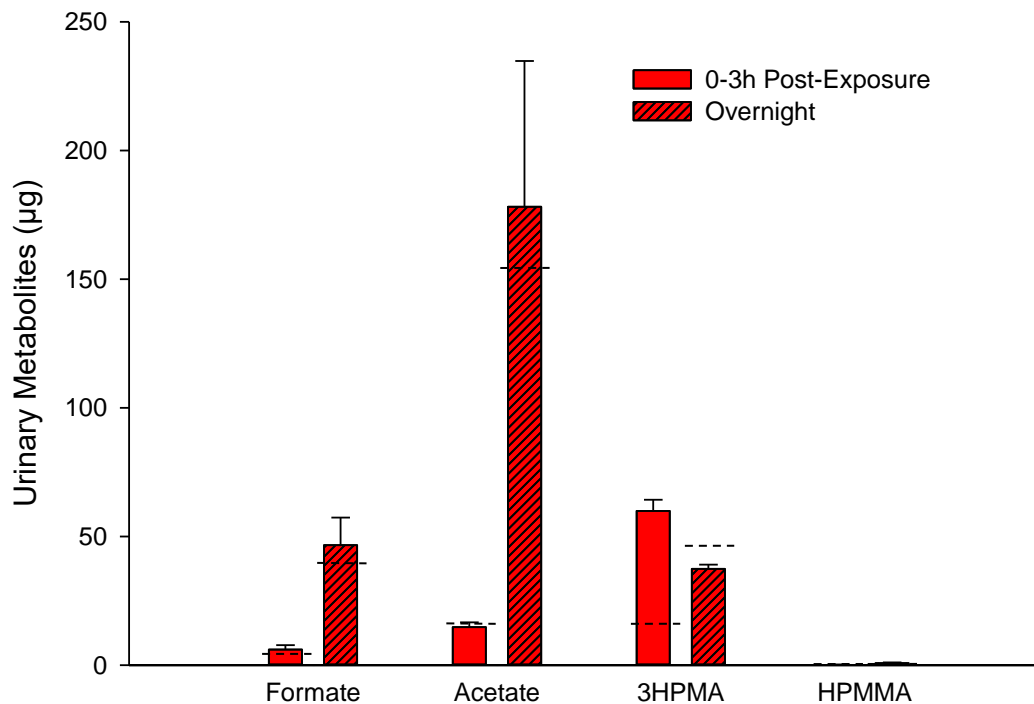


Fig. 4.8. Urinary metabolites after acute propylene glycol:vegetable glycerin (PG:VG; 50%:50%) exposure. Male mice were exposed (6h) to PG:VG (50%:50%) after which urine was collected at 0–1, 1–2, 2–3, and 3–16h (overnight) and aldehyde metabolites quantified. Values are mean \pm SE (n=5 mice per group). Dashed lines indicate metabolite measures (μg) after HEPA exposure (6h). **Abbr.:** **3HPMA**, 3-hydroxypropylmercapturic acid; **PG:VG**, propylene glycol:vegetable glycerin.

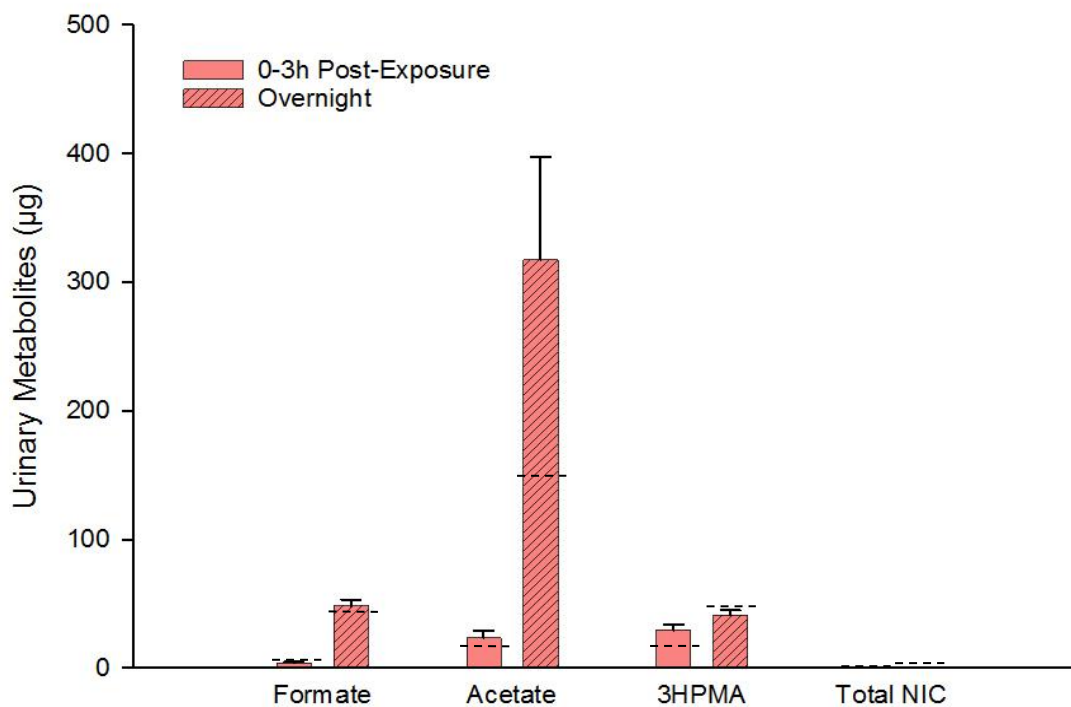


Fig. 4.9. Urinary metabolites after acute propylene glycol:vegetable glycerin (PG:VG; 30%:70%) exposure. Male mice were exposed (6h) to PG:VG (30%:70%) after which urine was collected at 0–1, 1–2, 2–3, and 3–16h (overnight) and aldehyde and tobacco alkaloid metabolites quantified. Values are mean \pm SE (n=4 mice per group). Dashed lines indicate metabolite measures (μg) after HEPA exposure (6h). **Abbr.:** **3HPMA**, 3-hydroxypropylmercapturic acid; **HPMMA**, 3-hydroxy-1-methylpropylmercapturic acid; **PG:VG**, propylene glycol:vegetable glycerin; **Total NIC**, total urinary nicotine metabolites (nicotine, cotinine, and trans-3'-hydroxycotinine).

than that measured after air exposure (Fig. 4.8). Levels of formate and acetate after the overnight (3-16h) collections were elevated compared with levels 0-3h post-exposure with either ratio of PG:VG; these elevations are likely related to generation by food, as mice were given free access to food during this collection period (Figs. 4.8 & 4.9).

10. Systemic Toxicity of Acute Exposure to Inhaled PG:VG

Because PG and VG, two humectants commonly used in e-cig devices, have been shown to generate measurable levels of aldehydes (168, 214-216), we tested for changes in systemic and hematological biomarkers in female mice exposed to PG:VG (30%:70%). PG:VG-exposed female mice showed a greater change in body weight during the exposure period (-2 g vs -1 g; $p=0.01$) but no changes in measured organ/body weight ratios (Table 4.7). Acute exposure had considerable effects on CBC measures. Exposed mice showed a significant decrease in leukocytes (-47%; $p=0.003$), driven by decreases in both neutrophils (-54%; $p=0.01$) and lymphocytes (-46%; $p=0.003$) (Table 4.8). Red blood cell levels were increased (+6%; $p<0.001$) as were measures of hemoglobin (+4%; $p=0.002$) and hematocrit (+6%; $p=0.002$) in PG:VG-exposed mice, while mean platelet volume (4%; $p=0.01$) and, modestly, mean corpuscular hemoglobin (-2%; $p=0.06$) were decreased (Table 4.8). Measures of systemic biomarkers showed no changes in muscle (CK) or LDH enzymes or creatinine but did show a significant increase in cholesterol (+15%; $p=0.03$) driven by an increase in LDL (+26%; $p=0.03$) (Table 4.7). Conversely, ALT was significantly decreased in exposed mice (-20%; $p=0.02$) (Table 4.7).

11. Acute Exposure to Inhaled PG:VG and Circulating Angiogenic Cells

Female mice exposed to 30%:70% PG:VG showed no change in the levels of CACs or Sca-1+ cells (Fig. 4.10A & B).

Table 4.7. Systemic parameters of female C57BL/6 mice acutely exposed to either air or propylene glycol:vegetable glycerin (PG:VG; 30%:70%).

| Variable | Exposure (4 days) | |
|------------------------------|-------------------|----------------|
| | Air Control | 30%:70% PG:VG |
| Change in BWT (g) | -1 ± 0 | -2 ± 0* |
| Heart/BWT (mg/g) | 5.2 ± 0.2 | 4.8 ± 0.1 |
| Lung/BWT (mg/g) | 6.4 ± 0.1 | 6.3 ± 0.2 |
| Liver/BWT (mg/g) | 38.8 ± 1.3 | 38.0 ± 0.6 |
| Kidney/BWT (mg/g) | 12.7 ± 0.3 | 11.8 ± 0.2 |
| Plasma Measurements | | |
| Cholesterol (mg/dL) | 44.74 ± 1.31 | 51.45 ± 1.77* |
| HDL (mg/dL) | 25.78 ± 1.05 | 30.36 ± 2.00 |
| LDL (mg/dL) | 12.80 ± 0.85 | 16.14 ± 0.47* |
| Triglycerides (mg/dL) | 30.81 ± 5.26 | 26.28 ± 1.54 |
| Albumin (g/dL) | 2.82 ± 0.07 | 2.98 ± 0.07 |
| Total Protein (g/dL) | 3.78 ± 0.11 | 3.94 ± 0.05 |
| ALT (U/I) | 20.20 ± 0.56 | 16.18 ± 0.34* |
| AST (U/I) | 64.10 ± 4.40 | 57.11 ± 4.33 |
| CK (U/I) | 164.16 ± 11.04 | 158.73 ± 24.29 |
| LDH (U/I) | 125.46 ± 8.91 | 127.20 ± 10.71 |
| Creatinine (mg/dL) | 0.21 ± 0.04 | 0.17 ± 0.01 |

Values = mean ± SE (n=5-10 mice per group); Abbr.: **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **BWT**, body weight; **CK**, creatine kinase; **HDL**, high-density lipoprotein; **LDH**, lactate dehydrogenase; **LDL**, low-density lipoprotein; **PG:VG**, propylene glycol:vegetable glycerin; *, p<0.05 compared with air control.

Table 4.8. Hematological measures of female C57BL/6 mice acutely exposed to either air or propylene glycol:vegetable glycerin (PG:VG; 30%:70%).

| Hematological Measurements | Exposure (4 days) | |
|--|-------------------|---------------|
| | Air Control | 30%:70% PG:VG |
| White Blood Cell (K/uL) | 1.34 ± 0.16 | 0.70 ± 0.05* |
| Neutrophils (K/uL) | 0.30 ± 0.06 | 0.14 ± 0.01* |
| Lymphocytes (K/uL) | 1.00 ± 0.11 | 0.54 ± 0.04* |
| Monocytes (K/uL) | 0.04 ± 0.01 | 0.02 ± 0.01 |
| Eosinophils (K/uL) | 0 ± 0 | 0 ± 0 |
| Basophils (K/uL) | 0 ± 0 | 0 ± 0 |
| Red Blood Cell (M/uL) | 8.73 ± 0.07 | 9.29 ± 0.07* |
| Hemoglobin (g/dL) | 12.1 ± 0.1 | 12.6 ± 0.1* |
| Hematocrit (%) | 44.9 ± 0.4 | 47.6 ± 0.5* |
| Mean Corpuscular Volume (fL) | 51.5 ± 0.5 | 51.2 ± 0.2 |
| Mean Corpuscular Hemoglobin (pg) | 13.9 ± 0.2 | 13.6 ± 0.1# |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 27.0 ± 0.3 | 26.5 ± 0.2 |
| Red Cell Distribution Width (%) | 17.0 ± 0.2 | 16.8 ± 0.1 |
| Platelets (K/uL) | 656 ± 27 | 710 ± 36 |
| Mean Platelet Volume (fL) | 4.4 ± 0 | 4.2 ± 0* |

Values = mean ± SE (n=10 mice per group); Abbr.: **PG:VG**, propylene glycol:vegetable glycerin; *, p<0.05 compared with air control;

#, 0.05≤p≤0.10 compared with air control.

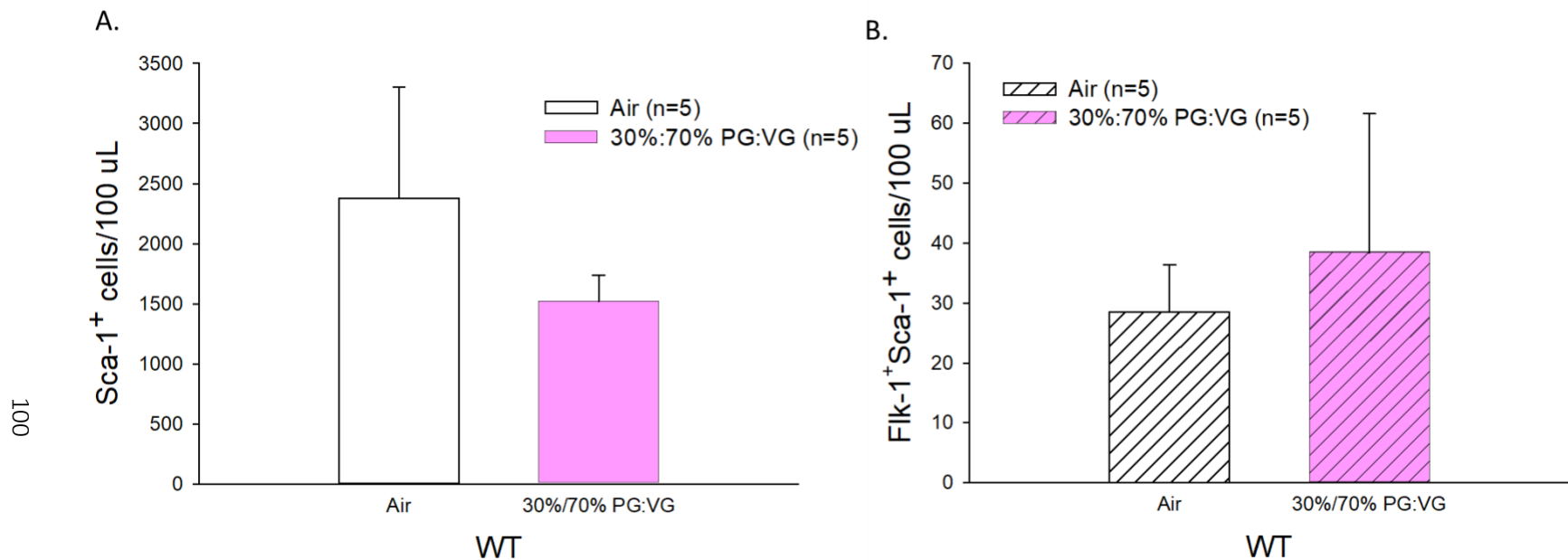


Fig. 4.10. Effects of acute propylene glycol:vegetable glycerin (PG:VG; 30%:70%) exposure on circulating stem cells in female mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in female C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or PG:VG (30%:70%, 6h/d) for 4 days. Levels of circulating Sca-1⁺ cells (**A**) and CACs (Flk-1⁺/Sca-1⁺ cells; **B**) were unchanged in PG:VG-exposed mice relative to air control mice. Values = mean \pm SE (n=5 mice per group).

D. Discussion

Tobacco smoking is the most significant modifiable risk factor in the development of CVD (14). As previously mentioned, tobacco smoke contains hundreds of thousands of compounds (396), and while the number of compounds generated in e-cig aerosols is still unknown, early analyses of the aerosol have shown that these devices still generate many of the harmful compounds found in MCS (96, 168, 400). The goal of this study was to examine the systemic, hematological, and endothelium-related effects of MCS, e-cig aerosol, and humectants used in e-cigs.

The results of these inhalation exposure studies revealed a number of important and novel observations and also confirmed findings previously described in the literature. Urinary analysis of male mice exposed to MCS showed that traditional cigarettes contain high levels of acrolein (measured via its urinary metabolite, 3HPMA), confirming previous data (114, 117, 147), but levels of other measured aldehyde metabolites are low in comparison. This may indicate that acrolein is responsible for the majority of the effects noted in response to MCS exposure; the significant role of acrolein in the toxicity of MCS has already been shown (145, 149). Exposure to JUUL VA Tobacco and Mango showed similar levels of urinary 3HPMA, suggesting that acrolein may also play a role in the health outcomes of these flavorings. However, exposure to aerosol from a JUUL device with either VA Tobacco, Mango, or Menthol flavoring significantly increased levels of total urinary nicotine metabolites (nicotine, cotinine, and 3HC). Nicotine, an important constituent in MCS, stimulates the sympathetic nervous system and has been shown to increase heart rate, blood pressure, platelet aggregation, myocardial contractility, and atherosclerotic development (401). JUUL devices use nicotine salts to deliver nicotine to users, and these salts allow for higher levels of nicotine delivery than other e-cig devices,

with one JUUL pod capable of providing a dose of nicotine similar to that of an entire pack of traditional cigarettes (402). Thus, it may be that the effects of JUUL devices are related to high levels of nicotine, although additional studies are needed to better understand the relationship between the levels of nicotine found in JUUL devices and our cardiovascular outcomes of interest.

Interestingly, although the levels of total urinary nicotine metabolites were increased in response to exposure to all of the flavored JUUL products compared with air-exposed control mice and MCS-exposed mice, levels were significantly higher after exposure to the mango and menthol flavorings than after exposure to VA Tobacco. This may suggest that the Mango and Menthol flavorings differ from VA Tobacco in some way that allows for greater nicotine exposure. Although there has not been much study of the JUUL Mango flavoring, menthol has long been used to provide a cooling or mint flavor in traditional cigarettes. Many studies have shown that the use of mentholated cigarettes allows for greater exposure to harmful constituents of MCS compared with the use of non-mentholated cigarettes (403, 404), whether through the attenuation of the respiratory irritation typically induced by MCS (405) or through decreased nicotine metabolism (406). The menthol flavoring used in JUUL devices may be inducing a similar effect and thus allowing for greater nicotine exposure. These differences in measures of urinary metabolites between flavors emphasizes the need to study individual e-liquid flavors to determine how they may induce different, specific health effects. Additionally, nationally representative survey data collected from approximately 14,000 middle and high school aged individuals showed that mint and mango were the most commonly used flavorings in JUUL devices (407), indicating the importance of further study of these flavors in

particular.

Analysis of the urinary metabolites in mice exposed to 30%:70% PG:VG or 50%:50% PG:VG showed significant increases in 3HPMA compared with baseline measures but varied in levels of formate and acetate. Thus, as with individual flavors, these data indicate the need to determine how varying ratios of PG:VG used in different e-cig devices may have varying health effects.

Acute exposure to MCS induced leukocyte suppression, driven by a decrease in lymphocytes. This is contrary to what is seen in epidemiological studies, where smokers show increased levels of leukocytes driven by increases in neutrophils, lymphocytes, and monocytes (345, 351-355). These changes are likely linked to the systemic inflammatory response activated by MCS exposure and the subsequent increase in the release of cells from the bone marrow (408, 409). The difference in outcomes may be due to the duration of our exposure; many epidemiological studies of smoking examine individuals who are chronic smokers and who may already have a significantly altered inflammatory response, while our mice are naïve animals never exposed to MCS or other air pollutants and whose response may be more similar to that seen in non-smokers.

In fact, analysis of more than 7,000 never-smoking adults from the third National Health and Nutrition Examination Survey found that participants with low but detectable cotinine levels—that is, individuals with secondhand smoke exposure—actually had decreased leukocyte levels compared with individuals without detectable cotinine levels (410). The authors of the study do not propose a hypothesis for why their leukocyte data are contrary to that seen in other epidemiological studies, but it does suggest that there are perhaps still nuances in the relationship between leukocytes and MCS exposure that need

to be explored, perhaps in relation to levels of circulating Sca-1⁺ cells, the hematological stem cell population from which leukocytes are derived. Acute MCS exposure in the current study did not change levels of CACs but did significantly decrease levels of Sca-1⁺ cells, and although the production of leukocytes occurs within the bone marrow, the decreased levels of Sca-1⁺ cells in the circulation may reflect decreased levels of these stem cells within the bone marrow as well, thus limiting leukocyte production. Additional studies of these stem cells in the bone marrow after MCS exposure could help clarify these outcomes.

Additional studies of the hematological response to MCS largely show that smoking increases several erythrocytic measures, including hemoglobin and platelets (345, 355, 411-413); levels of red blood cells are also often found to be increased (345, 414), although this is not always observed (355). Changes in erythrocytic measures, such as increased levels of hemoglobin, that are commonly seen in smokers are believed to be a compensatory mechanism to account for lower blood oxygen levels. Smoking introduces other gases such as carbon monoxide into the bloodstream where they can displace oxygen and lead to the activation of compensatory pathways that upregulate the production of hematological factors. Changes in mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration are also indices based on the composition of the hemoglobin found in red blood cells, and as hemoglobin changes in response to MCS exposure, so do these indices. The current MCS study confirms these findings; data reveal that the exposed mice have altered erythrocytic measures, showing significant changes in hemoglobin, hematocrit, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, as well as mean platelet volume, an indicator of

platelet size. The presence of these changes but not changes within leukocytes which are also commonly noted in smokers may indicate that changes in the hematological parameters occur earlier than those in leukocyte levels, although additional studies including chronic exposures to MCS are needed to confirm this hypothesis.

Electronic tobacco products such as e-cigs have not been in use for a period long enough to thoroughly determine the chronic outcomes of use, but acute studies using e-cig aerosol or various e-cig flavoring chemicals have shown that they have the potential to contribute to the development of CVD in a fashion similar to traditional cigarettes, including the development of endothelium dysfunction (190, 241), platelet activation and aggregation (370-372), and increases in markers of inflammation (415, 416). The results of our acute blu Plus+ e-cig exposure study largely agree with these early reports of e-cig toxicity. Leukocyte count was significantly decreased with exposure, driven by decreases in neutrophils, lymphocytes, and monocytes, and a handful of erythrocytic measures, including hemoglobin, were increased in response to exposure. Measures of endothelium function, however, did not indicate that blu Plus+ e-cig aerosol was contributing to dysfunction, as there was no change in the level of CACs in response to exposure. As there were also no change in CACs after MCS exposure, these outcomes may indicate that the endothelium dysfunction noted in response to exposure to these tobacco products takes longer than four days to develop. Nonetheless, the fact that MCS- and e-cig aerosol-exposures both show a number of similar outcomes that could contribute to the development of CVD may indicate that these outcomes are being driven by shared constituents, a premise that may be particularly important to the FDA as they seek to regulate ENDS.

Because risk for and prevalence of CVD have been shown to differ between men and women (417-422), we also examined the effects of tobacco product exposures on female mice. Like their MCS- and blu Plus+-exposed male counterparts, female mice acutely (4d) exposed to JUUL Mango showed a significant decrease in overall leukocyte count in addition to changes in erythrocytic measures, as did female mice acutely (4d) exposed to 30%:70% PG:VG. However, female mice exposed to JUUL Mango also showed modest decreases in levels of both Sca-1+ cells and CACs; although MCS exposure decreased the level of Sca-1+ cells in male mice, there was no change in CACs, and neither male mice exposed to blu Plus+ e-cig aerosol or female mice exposed to 30%:70% PG:VG showed changes in either cell type. Measures of systemic biomarkers showed that while female mice exposed to JUUL Mango only had minimal effects (modestly increased albumin and AST), female mice exposed to 30%:70% PG:VG had significantly increased cholesterol, driven by an increase in LDL. The risk of coronary heart disease has been correlated with levels of LDL (423), and a high level of cholesterol is a known risk factor for ischemic and coronary heart diseases (424). Although males are more likely than females to use traditional cigarettes (182, 325, 425, 426), and early reports of e-cig use suggest that males are also more likely than females to use these devices (182, 325, 427-429), there is preliminary data indicating that female smokers may have a greater prevalence of tobacco-related negative health outcomes than male smokers (426, 430). The change in Sca-1+ cells and CACs noted in female mice exposed to JUUL Mango may indicate a sex-dependent susceptibility to some component of this particular flavor, as these changes are not seen after exposure to 30%:70% PG:VG, the ratio of humectants commonly used in JUUL devices, in female mice. These data may also indicate that the

development of endothelium dysfunction is one of the earliest changes noted in response to exposure to JUUL Mango aerosol. Conversely, the change in cholesterol noted in 30%:70% PG:VG-exposed female mice may indicate that the e-cig humectants are first affecting systemic biomarkers rather than inducing changes in the endothelium. Additional studies are needed to determine whether these effects are seen in male mice exposed to 30%:70% PG:VG and JUUL Mango, to examine potential sex-dependent differences related to other e-cig flavors, and to better understand the vascular, hematological, and systemic effects of other e-cig flavors in general. Likewise, chronic exposure to both MCS and e-cig aerosols could reveal further sex-dependence in biomarkers of cardiovascular health

In summary, the studies presented in this chapter have demonstrated the impacts of traditional and emerging tobacco products on systemic and vascular health. In particular, these studies showed that exposure to e-cig aerosol (blu Plus+ and JUUL) produced levels of urinary aldehyde and tobacco alkaloid metabolites at levels similar to those found after MCS exposure, indicating that ENDS generate harmful and potentially harmful constituents much like MCS. Furthermore, data from systemic and hematological measures indicate that, like exposure to MCS, exposure to e-cig aerosol can significantly decrease levels of leukocytes, which could put users at a higher risk for infection. The decrease in CACs seen in female mice exposed to JUUL Mango but not 30%:70% PG:VG may indicate the potential for endothelium injury related to this specific flavor, but a comparable study in male mice exposed to JUUL Mango is needed to determine whether this specific outcome is sex-dependent. Likewise, the increase in cholesterol and LDL seen in female mice exposed to 30%:70% PG:VG may indicate the potential for significant

systemic effects known to contribute to the development of CVD in response to humectant exposure, but, again, a comparable study in male mice is needed to determine whether these outcomes are sex-dependent. As the use of emerging tobacco products continues to increase—particularly among youths—and as diseases such as EVALI continue to impact e-cig users, these studies provide novel insight into the health impacts of e-cigs and related components. Furthermore, these studies show similarities in exposure to aerosol from e-cigs and smoke from traditional cigarettes, whose roles in morbidity and mortality are well-known, and emphasize the need to study the health effects of e-cigs in both males and females in order to curtail another tobacco-related health epidemic.

CHAPTER V

SYSTEMIC, HEMATOLOGICAL, AND ENDOTHELIUM-RELATED EFFECTS OF FORMALDEHYDE AND ACETALDEHYDE IN MICE

A. Introduction

The saturated aldehydes formaldehyde (FR) and acetaldehyde (AA) are the smallest of the aldehydes. Both of these compounds can be found in a variety of food and drinks (99, 105, 106), and each is produced endogenously in small amounts (93, 97, 98, 105, 106). However, anthropogenic sources account for the majority of human exposure. FR is used in many consumer products, including carpets, manufactured wood products, paints, and preservatives (102), and both FR and AA are formed as products of combustion from industry, vehicle exhaust, and fires (101-103, 105, 106). Exposure to mainstream cigarette (MCS) or secondhand smoke can significantly increase the level of exposure to FR and AA. The World Health Organization estimates that smoking 20 cigarettes a day can increase exposure to FR up to 300% (99, 114, 117). Most AA exposure comes from food and drinks, so any inhalation can significantly increase exposure, with studies estimating that MCS can more than double AA exposure (107, 109, 117, 118). The Institute of Medicine ranks FR and AA as two of the most significant toxins in MCS, particularly in relation to cardiovascular disease (CVD) (96). The negative cardiovascular effects of FR and AA exposure have been previously documented. FR has been shown to cause

hematotoxicity, decreasing both red (93, 120, 122) and white (93, 120-122) blood cell and platelet counts (93, 120, 122) as well as levels of myeloid progenitor cells (120, 122). FR has also been linked to decreases in blood pressure, both via inhalation in rats (123) and ingestion in humans (128); *in vitro* studies show a concentration-dependent vascular relaxation in response to FR that supports these findings (124-127). Occupational exposures show also an association between increased exposure to FR and an increased incidence of heart disease (129-131). The cardiovascular effects of AA have largely been studied in relation to aldehyde dehydrogenase (ALDH2), the enzyme responsible for AA metabolism, and specifically the ALDH2 mutation that prevents AA metabolism, thus allowing blood levels of AA to increase (132). Increased blood levels of AA have been linked to a number of CVDs (132, 134-138) and changes in hematological factors (139-142).

Despite the significant exposure to FR and AA from MCS—as well as from electronic cigarette (e-cig) aerosol, as these devices have been shown to generate a variety of aldehydes (96, 180, 207-213)—and despite the high percent of the world’s population affected by mutated ALDH2 and who are thus more susceptible to AA exposure (133), few studies have addressed the effect of these compounds on endothelium function. Thus, the goal of these studies was to measure concentration- and duration-dependent as well as sex-dependent changes in biomarkers of vascular injury, specifically circulating angiogenic cells (CACs), and systemic and hematological biomarkers in mice exposed to FR and AA.

B. Experimental Procedures

1. Exposures

Animals were exposed to constituents via full-body inhalation as described in

Chapter II, Sections B.2. Upon completion of the exposure, mice were euthanized and tissues collected as described in Chapter II, Section B.5.

2. Urine Collection

Urine samples were collected post-exposure as described in Chapter II, Section B.3.

3. Urine Metabolite Quantification

Collected urine was used to quantify levels of volatile organic compounds (VOC), saturated aldehyde, and tobacco alkaloid metabolites using analytical methods as described in Chapter II, Section B.4.

4. Complete Blood Cell Count (CBC)

A sample of whole blood was collected after the final day of exposure for a complete blood cell count (CBC) as described in Chapter II, Section B.6.

5. Plasma Biomarkers Quantification

Plasma was prepared from each sample for an analysis of a number of biomarkers as described in Chapter II, Section B.7.

6. Circulating Angiogenic Cell (CAC) Quantification

Flow cytometry was used to analyze the number of CACs in each sample as described in Chapter II, Section B.8.

7. Statistical Analyses

Statistical analyses were performed as described in Chapter II, Section B.10.

C. Results

1. Urinary Formaldehyde Metabolite in MCS-, PG:VG-, and Formaldehyde-Exposed Mice

Although exposure to FR occurs frequently through ingestion of various food

products or by exposure to exhaust and other combustion products (100-104), the use of tobacco products results in a much greater level of exposure to FR (93, 95, 99). Furthermore, although formate, the primary urinary metabolite of FR, has not been validated as a urinary biomarker of tobacco exposure (431, 432), studies have shown that it can be used in conjunction with other biomarkers as an indicator of tobacco product use or exposure (168, 293, 433). To better understand how FR-alone exposure related to MCS exposure from a known number of cigarettes and to e-cig exposure from known ratios of PG:VG, we measured urinary formate by UPLC-MS/MS to confirm that the concentration of FR used in our FR-alone exposure was comparable to exposures from real-world sources. For this, male mice were exposed to FR (5 ppm, 6h), MCS (50% of smoke of 12 KY Reference 3R4F cigarettes, 6h), or PG:VG (50%:50% or 30%:70%, 6h), and urine was collected immediately after the exposure sessions.

Urinary formate levels in the first three hours post-exposure were not significantly different between mice exposed to FR (5 ppm) or to 30%:70% PG:VG compared with baseline, but mice exposed to MCS or 50%:50% PG:VG showed significantly increased levels ($p=0.009$ and $p=0.003$, respectively) (Fig. 5.1). Additionally, levels of formate in MCS-exposed mice were significantly higher than levels in FR-exposed mice (Fig. 5.1). Formate levels in FR-exposed mice were not significantly different from those in PG:VG-exposed mice, likely indicating that the concentration of FR generated for the FR-alone exposure (5 ppm) is similar to the levels generated in PG:VG, regardless of the ratio (Fig. 5.1). Levels of formate after the overnight (3-16h) collections were elevated compared with levels 0-3h post-exposure with all exposures; these elevations are likely related to generation by food, as mice were given free access to food during this collection period

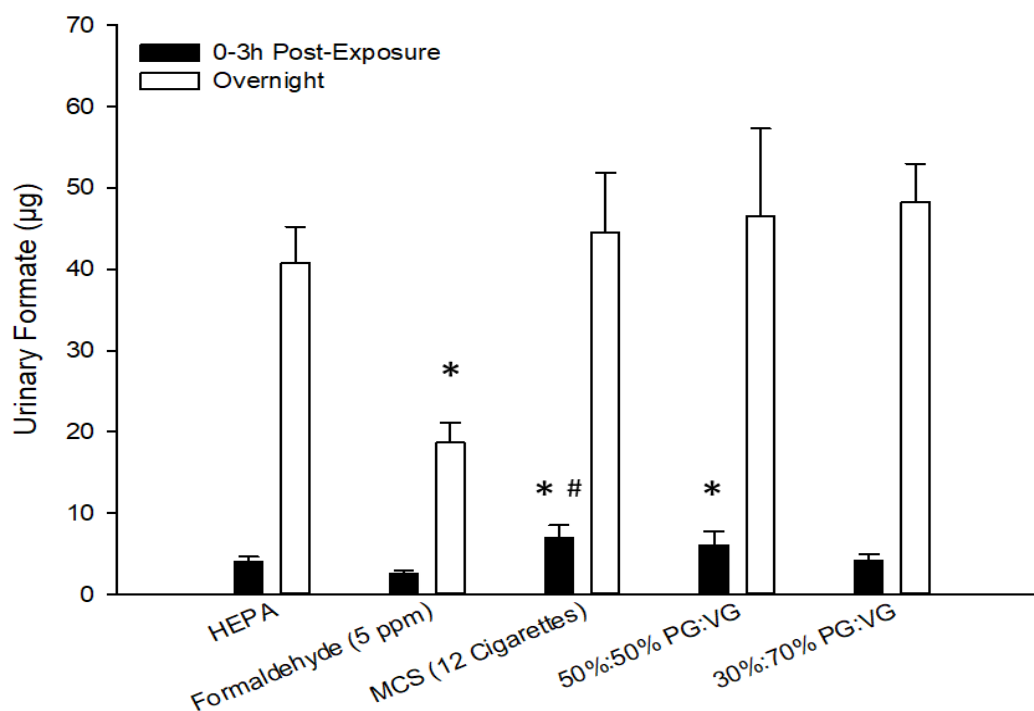


Fig. 5.1. Urinary formate levels in formaldehyde- or tobacco product-exposed male mice. Male mice were exposed (6h) to formaldehyde (5 ppm), mainstream cigarette smoke (MCS; 50% smoke of 12 cigarettes), or propylene glycol:vegetable glycerin (PG:VG; 50%:50% or 30%:70%, 6h) after which urine was collected at 0–1, 1–2, 2–3, and 3–16h (overnight) and formate quantified. Values are mean \pm SE (n=4-12 mice per group). *, p<0.05 compared with HEPA; #, p<0.05 compared with formaldehyde.

(Fig. 5.1). Additionally, mice exposed to FR had lower levels of formate after the overnight exposure compared with baseline (Fig. 5.1).

2. Systemic Toxicity of Two-Week Exposure to Inhaled Formaldehyde

Because of the potential for MCS to significantly increase levels of FR exposure (99, 114, 117), we tested for changes in systemic and hematological biomarkers in mice exposed to FR (5 ppm). Body weight and organ/body weight ratios showed significant changes between the air- and FR-exposed groups; exposure to 5 ppm FR caused decreases in final body weight (-8%; $p=0.02$) and the liver/body weight ratio (-5%; $p=0.08$) and an increase in the lung/body weight ratio (+12%, $p=0.002$) (Table 5.1). Two weeks of exposure had modest to considerable effects on CBC and plasma biomarkers. Exposed mice had a decreased level of leukocytes (-46%; $p=0.004$), driven by decreases in all leukocyte subpopulations, but an increased level of red blood cells (+9%; $p=0.01$) (Table 5.2). Likewise, hemoglobin levels (+7%; $p=0.02$) were increased, while mean corpuscular hemoglobin concentration (-2%; $p=0.08$), platelet count (-17%; $p=0.01$), and mean platelet volume (-12%; $p=0.002$) were all decreased (Table 5.2). A low volume of plasma limited additional measurements of systemic toxicity, but exposed mice showed a significant decrease in cholesterol compared with air-exposed control mice (-32%; $p=0.03$) (Table 5.1).

3. Two-Week Exposure to Formaldehyde and Circulating Angiogenic Cells

Decreased levels of CACs have been associated with increased CVD risk, and it is thought that CAC levels serve as a predictor of overall cardiovascular health and future cardiovascular events (66). Altered CAC levels are noted in individuals exposed to secondhand smoke (72) and during smoking cessation (318). Like the changes seen in

Table 5.1. Systemic parameters of male C57BL/6 mice acutely exposed to either air or formaldehyde (FR; 1, 2, or 5 ppm).

| Variable | Exposure | | | | |
|------------------------------|----------------|-----------------|----------------|-----------------|---------------|
| | Air Control | 4 days | | | 2 weeks |
| | | FR (1 ppm) | FR (2 ppm) | FR (5 ppm) | FR (5 ppm) |
| Final BWT (g) | 28 ± 0 | 29 ± 0 | 29 ± 1 | 27 ± 1 | 26 ± 0* |
| Heart/BWT (mg/g) | 5.0 ± 0.1 | 5.0 ± 0.3 | 4.9 ± 0.1 | 5.1 ± 0.2 | 4.8 ± 0.1 |
| Lung/BWT (mg/g) | 5.6 ± 0.1 | 5.1 ± 0.2 | 5.2 ± 0.1 | 5.5 ± 0.2 | 6.2 ± 0.1* |
| Liver/BWT (mg/g) | 42.1 ± 0.6 | 42.9 ± 1.0 | 42.3 ± 0.8 | 40.5 ± 1.2 | 40.2 ± 1.0 |
| Kidney/BWT (mg/g) | 12.9 ± 0.2 | 12.6 ± 0.2 | 12.2 ± 0.2* | 11.2 ± 0.8* | 13.1 ± 0.3 |
| Plasma Measurements | | | | | |
| Cholesterol (mg/dL) | 96.03 ± 3.67 | 117.89 ± 2.64* | 103.83 ± 3.13 | 94.23 ± 2.32 | 65.45 ± 5.29* |
| HDL (mg/dL) | 66.31 ± 4.33 | N/A | N/A | 67.88 ± 2.90 | N/A |
| Triglycerides (mg/dL) | 47.84 ± 2.20 | 44.57 ± 2.22 | 33.75 ± 3.86* | 43.64 ± 2.01 | 50.48 ± 2.15 |
| Albumin (g/dL) | 3.37 ± 0.06 | 3.71 ± 0.05* | 3.66 ± 0.05* | 3.21 ± 0.06 | N/A |
| Total Protein (g/dL) | 4.74 ± 0.08 | 4.50 ± 0.07# | 4.67 ± 0.05 | 5.18 ± 0.13* | N/A |
| ALT (U/I) | 24.40 ± 1.65 | 19.99 ± 0.71 | 24.84 ± 1.82 | 38.44 ± 7.75# | N/A |
| AST (U/I) | 77.33 ± 7.55 | 40.63 ± 2.72* | 60.61 ± 3.96 | 104.26 ± 14.34* | N/A |
| CK (U/I) | 277.82 ± 26.67 | 169.65 ± 12.31* | 267.89 ± 34.08 | 314.13 ± 68.39 | N/A |
| LDH (U/I) | 230.89 ± 21.17 | 156.74 ± 7.25* | 263.01 ± 24.92 | 188.06 ± 23.20 | N/A |
| Creatinine (mg/dL) | 0.31 ± 0.01 | 0.30 ± 0.01 | 0.38 ± 0.01* | 0.37 ± 0.01* | N/A |

Values = mean ± SE (n=6-11 mice per group); Abbr.: **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **BWT**, body weight; **CK**, creatine kinase; **FR**, formaldehyde; **HDL**, high-density lipoprotein; **LDH**, lactate dehydrogenase; *, p<0.05 compared with air control; #, 0.05≤p≤0.10 compared with air control.

Table 5.2. Hematological measures of male C57BL/6 mice acutely exposed to either air or formaldehyde (FR; 1, 2, or 5 ppm).

| Hematological Measurements | Air Control | 4 days | | | 2 weeks |
|--|-------------|--------------|-------------|--------------|---------------|
| | | FR (1 ppm) | FR (2 ppm) | FR (5 ppm) | FR (5 ppm) |
| White Blood Cell (K/uL) | 2.35 ± 0.18 | 2.11 ± 0.23 | 2.06 ± 0.10 | 2.94 ± 0.49 | 1.27 ± 0.15* |
| Neutrophils (K/uL) | 0.66 ± 0.11 | 0.27 ± 0.03* | 0.46 ± 0.04 | 0.79 ± 0.16 | 0.31 ± 0.05# |
| Lymphocytes (K/uL) | 1.60 ± 0.12 | 1.71 ± 0.19 | 1.44 ± 0.11 | 2.07 ± 0.40 | 0.92 ± 0.11* |
| Monocytes (K/uL) | 0.07 ± 0.01 | 0.08 ± 0.02 | 0.06 ± 0.01 | 0.06 ± 0.01 | 0.04 ± 0.01* |
| Eosinophils (K/uL) | 0.01 ± 0 | 0.06 ± 0.01* | 0.01 ± 0 | 0.01 ± 0.01 | 0 ± 0# |
| Basophils (K/uL) | 0.01 ± 0 | 0 ± 0 | 0 ± 0 | 0.01 ± 0 | 0 ± 0* |
| Red Blood Cell (M/uL) | 9.54 ± 0.16 | 9.49 ± 0.13 | 9.13 ± 0.26 | 8.45 ± 0.17* | 10.39 ± 0.11* |
| Hemoglobin (g/dL) | 13.6 ± 0.2 | 13.6 ± 0.1 | 12.3 ± 0.3* | 12.3 ± 0.2# | 14.6 ± 0.1* |
| Hematocrit (%) | 41.3 ± 0.7 | 45.1 ± 0.5* | 39.0 ± 1.1# | 35.0 ± 0.7* | 43.9 ± 0.5 |
| Mean Corpuscular Volume (fL) | 43.3 ± 0.3 | 47.5 ± 0.3* | 42.7 ± 0.2 | 41.5 ± 0.3* | 42.2 ± 0.4 |
| Mean Corpuscular Hemoglobin | 14.2 ± 0.1 | 14.3 ± 0.1 | 13.6 ± 0.2* | 14.6 ± 0.1# | 14.0 ± 0.1# |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 32.9 ± 0.3 | 30.1 ± 0.2* | 31.7 ± 0.3 | 35.3 ± 0.4* | 33.2 ± 0.3 |
| Red Cell Distribution Width (%) | 18.4 ± 0.2 | N/A | 17.6 ± 0.2* | 17.6 ± 0.4 | 17.9 ± 0.3 |
| Platelets (K/uL) | 735 ± 24 | 800 ± 16 | 810 ± 34 | 715 ± 30 | 612 ± 55* |
| Mean Platelet Volume (fL) | 4.8 ± 0.1 | 6.0 ± 0* | 4.7 ± 0.1 | 4.5 ± 0 | 4.3 ± 0.1* |

Values = mean ± SE (n=6-11 mice per group); Abbr.: **FR**, formaldehyde; *, p<0.05 compared with air control; #, 0.05≤p≤0.10 compared with air control.

response to tobacco smoke, there was a significant decrease in the level of CACs (Flk-1+/Sca-1+ cells) after two weeks of FR exposure (5 ppm; -56%, $p=0.003$) (Fig. 5.2B). Levels of Sca-1+ cells, the larger hematopoietic stem cell population from which CACs are derived, were also significantly decreased compared with air-exposed control mice (-50%; $p=0.01$) (Fig. 5.2A).

4. Systemic Toxicity of Acute Exposure to Inhaled Formaldehyde

Because two weeks of exposure to FR (5 ppm) had specific systemic toxicity that included changes in body weight, CBC (leukocytes, red blood cells, hemoglobin), plasma factors (cholesterol), and CACs (decreased), we performed acute FR exposures to better understand the temporal relationships between these factors. Thus, we tested whether acute FR exposures would induce rapid toxicity that was otherwise compensated for in a longer setting. Acute exposure of male mice (1, 2, or 5 ppm; 4 days) had limited effects on organ and body weights (or ratios) or in leukocytes, but caused significant changes in erythrocytes and related measures (Tables 5.1 & 5.2). Exposure to a higher concentration of FR (5 ppm) caused no change in overall body weight but induced a significant decrease in kidney/body weight ratio (-13%; $p=0.001$) (Table 5.1). Although the overall leukocyte count and counts of subpopulations were unchanged, FR-exposed mice showed decreased red blood cell count (-11%; $p=0.003$), hemoglobin (-9%; $p=0.10$), hematocrit (-15%; $p<0.001$), and mean corpuscular volume (-4%; $p=0.003$) but increased mean corpuscular hemoglobin (+3%; $p=0.06$) and mean corpuscular hemoglobin concentration (+7%; $p=0.002$) (Table 5.2). Acute exposure to FR (5 ppm) did not affect plasma levels of CK (striated muscle) or LDH (non-specific cell toxicity) enzymes, but did increase levels of plasma total protein (+9%; $p=0.01$), creatinine (+20%; $p=0.03$), AST (liver) (+35%;

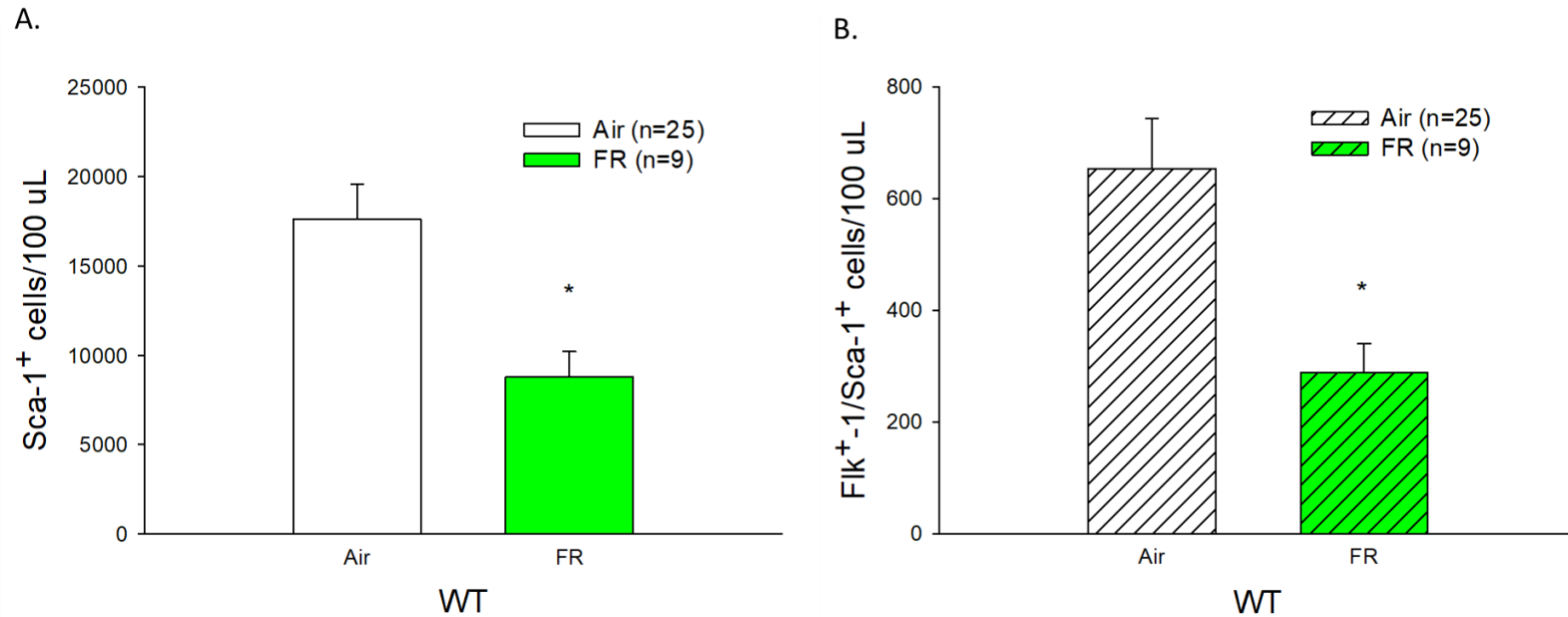


Fig. 5.2. Effects of two-week formaldehyde (FR; 5 ppm) exposure on circulating stem cells in male mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in male C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or formaldehyde (5 ppm, 6h/d) for two weeks. After two weeks of exposure to FR, the levels of both circulating Sca-1⁺ cells (**A**) and CACs (Flk-1⁺/Sca-1⁺ cells) (**B**) were significantly decreased in WT mice. Values = mean ± SE (n= 9-25 mice per group). *, p<0.05 compared with air control.

p=0.03), and, modestly, ALT (liver) (+58%; p=0.07) compared with the air-exposed control group (Table 5.1).

Unsurprisingly, acute (4d) exposure to FR at lower concentrations (1 and 2 ppm) showed similar but diminished effects overall on markers of systemic and hematological toxicity than 5 ppm exposure. Exposure to FR (2 ppm) showed a similar decrease in kidney/body weight ratio (-6%; p=0.04) but no changes in leukocytes (Tables 5.1 & 5.2). Measures of hemoglobin and hematocrit were likewise decreased (-9%; p=0.02 and -6%; p=0.08, respectively), but red cell distribution width was also increased (-4%; p=0.02), while mean corpuscular hemoglobin was decreased rather than increased as was seen at a higher FR concentration (5 ppm) (-5%; p<0.001) (Table 5.2). Creatinine was again increased (+23%; p<0.001), and changes in triglycerides (-29%; p=0.003) and albumin (+9%; p=0.002) were also induced at this level of exposure (2 ppm) (Table 5.1). At the lowest-tested concentration of FR (1 ppm), acute (4d) exposure caused no changes in organ or body weights (or ratios) (Table 5.1). Plasma levels of triglycerides, ALT, and creatinine remained unchanged, however, levels of plasma cholesterol and albumin were increased (+23%; p=0.004 and +10%; p=0.01, respectively), while levels of plasma total protein (-5%; p=0.08), AST (-47%; p=0.001), CK (-39%; p=0.04), and LDH (-32%; p=0.01) were decreased (Table 5.1). Although there was no change in overall leukocyte count, neutrophil count was decreased (-60%; p=0.04) and eosinophils increased (+438%; p<0.001) (Table 5.2). Contrary to changes seen at higher FR concentrations (2 and/or 5 ppm), both hematocrit (+9%; p=0.01) and mean corpuscular volume (+10%; p<0.001) were increased and mean corpuscular hemoglobin concentration decreased (-8%; p<0.001) compared with air-exposed control mice; mean platelet volume was also increased with

low level (1 ppm) FR exposure (+24%; p=0.002) (Table 5.2).

In female mice, acute exposure to FR (2 or 5 ppm; 4 days) had limited to modest effects on organ and body weights (or ratios), CBC, or plasma biomarkers (Tables 5.3 & 5.4). For example, female mice acutely exposed to 2 ppm FR had no change in overall body weight or organ/body weight ratios (Table 5.3) and only increases in eosinophils (+231%; p=0.01) and basophils (+141%; p=0.09) (Table 5.4). Plasma levels in liver (ALT, AST) and CK (muscle) enzymes and creatinine were unchanged, but LDH and, modestly, albumin were increased (+37%; p=0.03 and +6%; p=0.09, respectively) (Table 5.3). Surprisingly, 4 days of FR (5 ppm) exposure in female mice has a similarly minimal effect on markers of systemic toxicity. Cholesterol (-32%; p=0.02), triglycerides (-14%; p=0.05), albumin (-14%; p=0.01), and total protein (-9%; p=0.03) were all decreased, as were monocyte levels (-37%; p=0.04) and mean platelet volume (-9%; p=0.004) (Table 5.3).

5. Acute Exposure to Formaldehyde and Circulating Angiogenic Cells

Acute (4d) exposure to 5 ppm FR increased the levels of CACs in male mice (+62%; p=0.002), but there was no change in the level of Sca-1⁺ cells (Fig. 5.3A & B). Acute (4d) exposure to 2 ppm FR did not change the level of Sca-1⁺ cells or CACs (Fig. 5.4A & B). Likewise, female mice had no change in the levels of CACs or Sca-1⁺ cells with acute FR (2 or 5 ppm) exposure (Figs. 5.5A & B and 5.6A & B).

6. Urinary Acetaldehyde Metabolite in MCS-, PG:VG-, and Acetaldehyde-Exposed Mice

Although exposure to AA occurs largely through the ingestion of various food products, particularly via alcohol consumption (105-107), the use of tobacco products can result in a much greater level of exposure to AA (93, 95, 99). Like formate, acetate, the

Table 5.3. Systemic parameters of female C57BL/6 mice acutely exposed to either air or formaldehyde (FR; 2 or 5 ppm).

| Variable | Exposure | | |
|----------------------------|----------------|-----------------|----------------|
| | Air Control | FR (2 ppm) | FR (5 ppm) |
| Final BWT (g) | 26 ± 1 | 25 ± 1 | 26 ± 1 |
| Heart/BWT (mg/g) | 4.8 ± 0.1 | 5.0 ± 0.1 | N/A |
| Lung/BWT (mg/g) | 7.0 ± 0.3 | 6.6 ± 0.2 | N/A |
| Liver/BWT (mg/g) | 43.5 ± 1.0 | 41.9 ± 1.7 | N/A |
| Kidney/BWT (mg/g) | 11.5 ± 0.2 | 11.8 ± 0.2 | N/A |
| Plasma Measurements | | | |
| Cholesterol (mg/dL) | 69.58 ± 5.64 | 78.89 ± 3.13 | 48.09 ± 4.01* |
| HDL (mg/dL) | 62.82 ± 2.73 | 55.89 ± 3.15 | N/A |
| Triglycerides (mg/dL) | 31.17 ± 2.04 | 35.76 ± 3.51 | 26.83 ± 1.82# |
| Albumin (g/dL) | 3.03 ± 0.08 | 3.21 ± 0.06# | 2.60 ± 0.06* |
| Total Protein (g/dL) | 4.93 ± 0.23 | 5.17 ± 0.10 | 4.47 ± 0.20* |
| ALT (U/I) | 20.32 ± 1.31 | 21.24 ± 1.43 | 16.77 ± 1.29 |
| AST (U/I) | 77.51 ± 5.80 | 94.87 ± 13.23 | 77.62 ± 15.65 |
| CK (U/I) | 240.68 ± 33.97 | 318.58 ± 46.05 | 229.07 ± 50.25 |
| LDH (U/I) | 158.19 ± 13.62 | 217.01 ± 28.28* | 139.31 ± 15.86 |
| Creatinine (mg/dL) | 0.29 ± 0.02 | 0.32 ± 0.01 | 0.25 ± 0.01 |

Values = mean ± SE (n=6-10 mice per group); Abbr.: **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **BWT**, body weight; **CK**, creatine kinase; **FR**, formaldehyde; **HDL**, high-density lipoprotein; **LDH**, lactate dehydrogenase; *, p<0.05 compared with air control; #, 0.05≤p≤0.10 compared with air control.

Table 5.4. Hematological measures of female C57BL/6 mice acutely exposed to either air or formaldehyde (FR; 2 or 5 ppm).

| Hematological Measurements | Exposure | | |
|--|-------------|--------------|--------------|
| | Air Control | FR (2 ppm) | FR (5 ppm) |
| White Blood Cell (K/uL) | 2.20 ± 0.26 | 2.24 ± 0.24 | 2.13 ± 0.34 |
| Neutrophils (K/uL) | 0.55 ± 0.07 | 0.57 ± 0.07 | 0.75 ± 0.20 |
| Lymphocytes (K/uL) | 1.57 ± 0.21 | 1.54 ± 0.18 | 1.34 ± 0.19 |
| Monocytes (K/uL) | 0.08 ± 0.01 | 0.09 ± 0.01 | 0.05 ± 0.01* |
| Eosinophils (K/uL) | 0.01 ± 0.01 | 0.03 ± 0.01* | 0 ± 0 |
| Basophils (K/uL) | 0 ± 0 | 0.01 ± 0.01# | 0 ± 0 |
| Red Blood Cell (M/uL) | 8.46 ± 0.23 | 8.68 ± 0.27 | 8.93 ± 0.20 |
| Hemoglobin (g/dL) | 12.2 ± 0.4 | 13.0 ± 0.3 | 12.1 ± 0.2 |
| Hematocrit (%) | 35.9 ± 1.0 | 37.6 ± 1.1 | 37.5 ± 0.7 |
| Mean Corpuscular Volume (fL) | 42.5 ± 0.3 | 43.3 ± 0.4 | 42.0 ± 0.4 |
| Mean Corpuscular Hemoglobin | 14.5 ± 0.3 | 15.0 ± 0.2 | 13.6 ± 0.2 |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 34.0 ± 14.0 | 34.5 ± 0.3 | 32.3 ± 0.2 |
| Red Cell Distribution Width (%) | 17.5 ± 0.2 | 17.5 ± 0.2 | 17.6 ± 0.2 |
| Platelets (K/uL) | 803 ± 45 | 737 ± 31 | 684 ± 49 |
| Mean Platelet Volume (fL) | 4.9 ± 0.1 | 4.9 ± 0.1 | 4.4 ± 0.1* |

Values = mean ± SE (n=6-10 mice per group); Abbr.: **FR**, formaldehyde; *, p<0.05 compared with air control; #, 0.05≤p≤0.10 compared with air control.

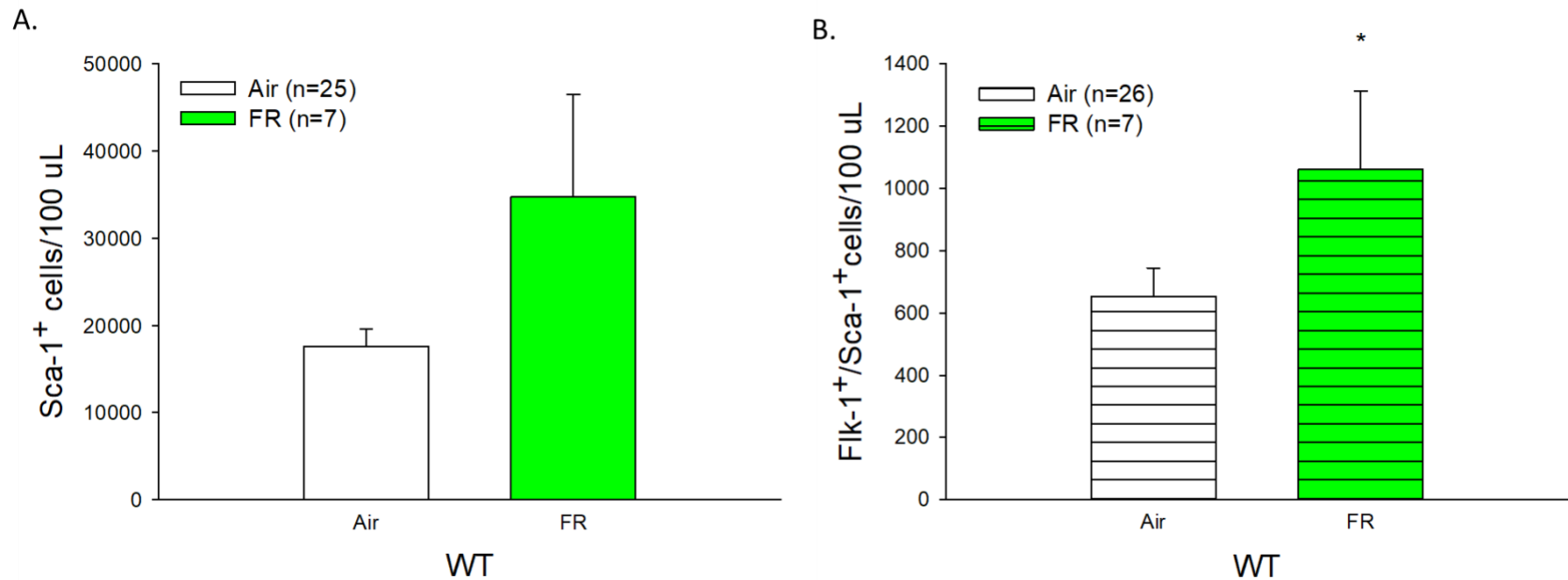


Fig. 5.3. Effects of acute formaldehyde (FR; 5 ppm) exposure on circulating stem cells in male mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in male C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or formaldehyde (5 ppm, 6h/d) for 4 days. After four days of exposure to FR, the levels of circulating Sca-1⁺ cells were unchanged (**A**), however, the level of CACs (Flk-1⁺/Sca-1⁺ cells) was significantly increased in WT mice (**B**). Values = mean ± SE (n= 7-26 mice per group). *, p<0.05 compared with air control.

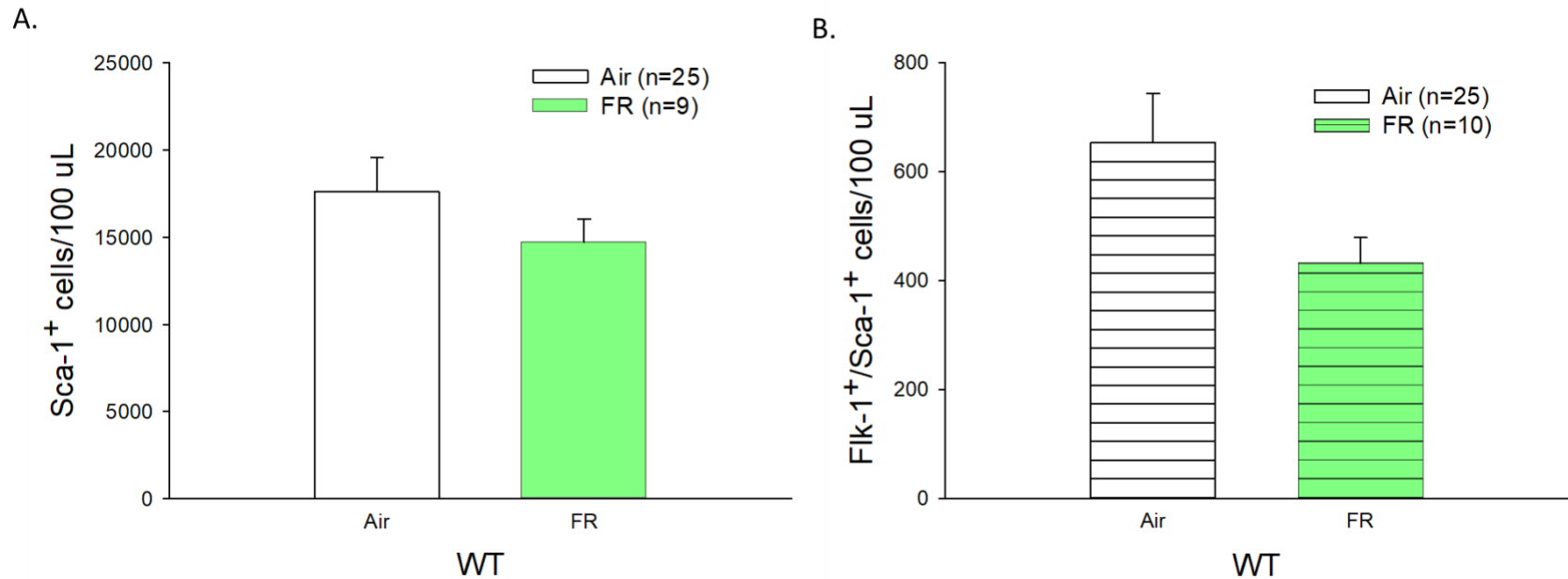


Fig. 5.4. Effects of acute formaldehyde (FR; 2 ppm) exposure on circulating stem cells in male mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in male C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or formaldehyde (2 ppm, 6h/d) for 4 days. Levels of circulating Sca-1⁺ cells (A) and CACs (Flk-1⁺/Sca-1⁺ cells; B) were unchanged in FR-exposed mice relative to air control mice. Values = mean \pm SE (n=9-25 mice per group).

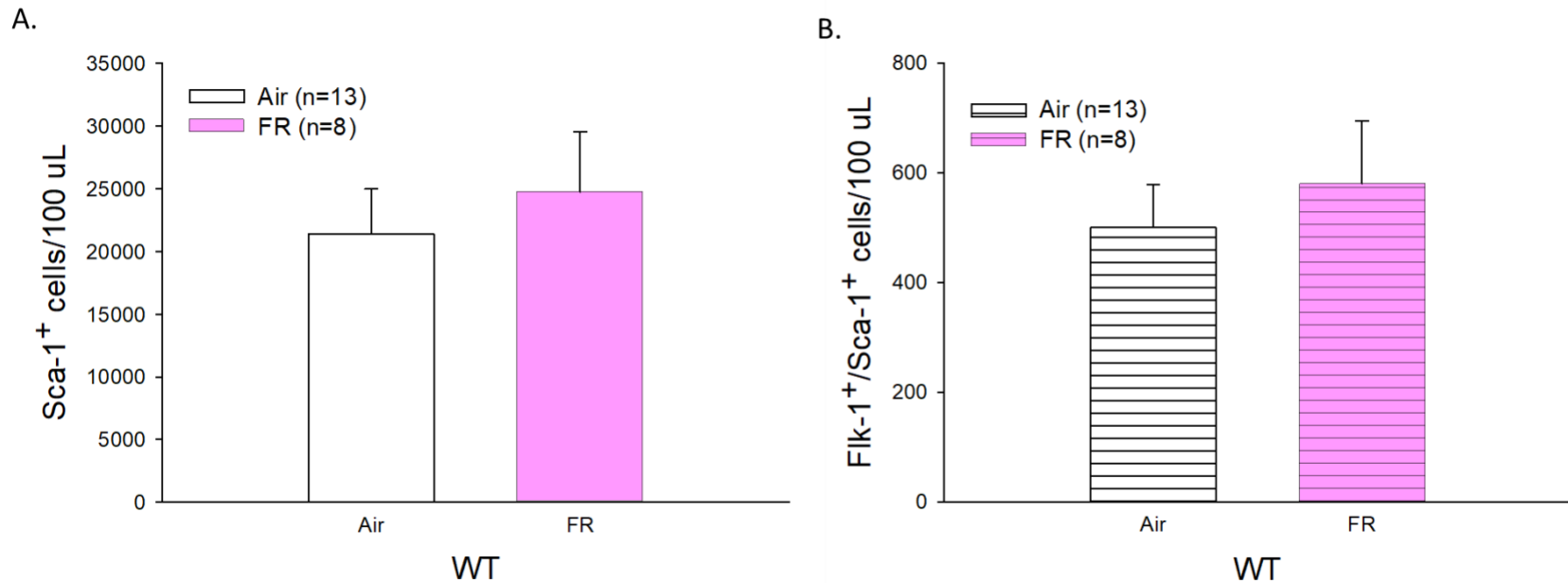


Fig. 5.5. Effects of acute formaldehyde (FR; 2 ppm) exposure on circulating stem cells in female mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in female C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or formaldehyde (2 ppm, 6h/d) for 4 days. Levels of circulating Sca-1⁺ cells (A) and CACs (Flk-1⁺/Sca-1⁺ cells; B) were unchanged in FR-exposed mice relative to air control mice. Values = mean \pm SE (n=8-13 mice per group).

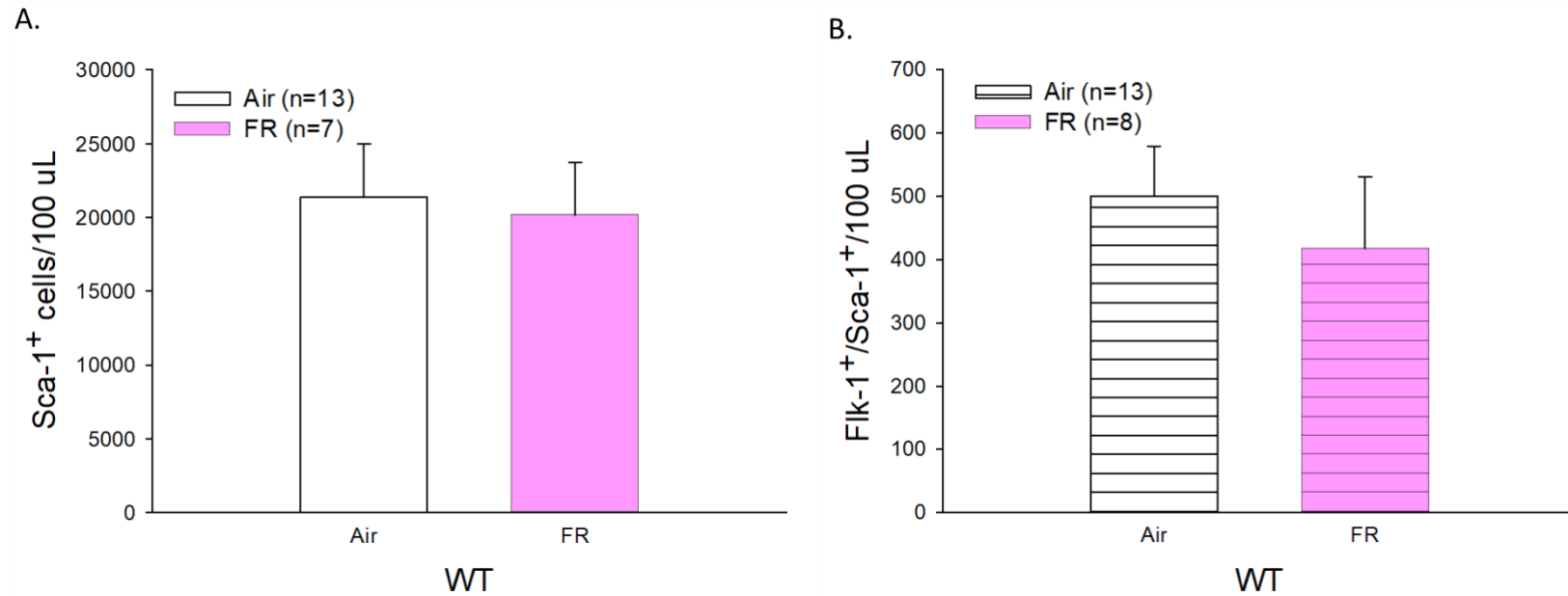


Fig. 5.6. Effects of acute formaldehyde (FR; 5 ppm) exposure on circulating stem cells in female mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in female C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or formaldehyde (5 ppm, 6h/d) for 4 days. Levels of circulating Sca-1⁺ cells (A) and CACs (Flk-1⁺/Sca-1⁺ cells; B) were unchanged in FR-exposed mice relative to air control mice. Values = mean \pm SE (n=7-13 mice per group).

primary urinary metabolite of AA, has not been validated as a urinary biomarker of tobacco exposure (431, 432), but it has been shown to increase with tobacco-product use (168, 293, 434). To better understand how exposure to AA-alone related to MCS exposure from a known number of cigarettes and to e-cig exposure from known ratios of PG:VG, we measured urinary acetate by UPLC-MS/MS to confirm that the concentration of AA used in our AA-alone exposure was comparable to exposures from real-world sources. For this, male mice were exposed to AA (5 ppm, 6h), MCS (50% of smoke of 12 KY Reference 3R4F cigarettes 6h), or PG:VG (50%:50% or 30%:70%, 6h), and urine was collected immediately after the exposure sessions. Measures of urinary acetate in AA-, MCS-, and 50%:50% PG:VG-exposed mice were not significantly different from baseline during the first three hours post-exposed, but mice exposed to 30%:70% PG:VG showed increased levels ($p=0.047$) (Fig. 5.7). As the levels of acetate in AA-exposed mice were not significantly different from MCS-exposed mice, this likely indicates that the concentration of AA generated for the AA-alone exposure (5 ppm) is similar to the level generated in MCS (Fig. 5.7). Levels of acetate after the overnight (3-16h) collections were elevated compared with levels 0-3h post-exposure with all exposures; these elevations are likely related to generation by food, as mice were given free access to food during this collection period (Fig. 5.7). Additionally, levels of acetate during the overnight collection were significantly higher in 30%:70% PG:VG-exposed mice compared with baseline (Fig. 5.7).

7. Systemic Toxicity of Two-Week Exposure to Inhaled Acetaldehyde

Because of the potential for MCS to significantly increase levels of AA exposure (107, 118), we tested for changes in systemic and hematological biomarkers in mice exposed to AA (5 ppm). Two weeks of exposure had limited to modest effects on organ

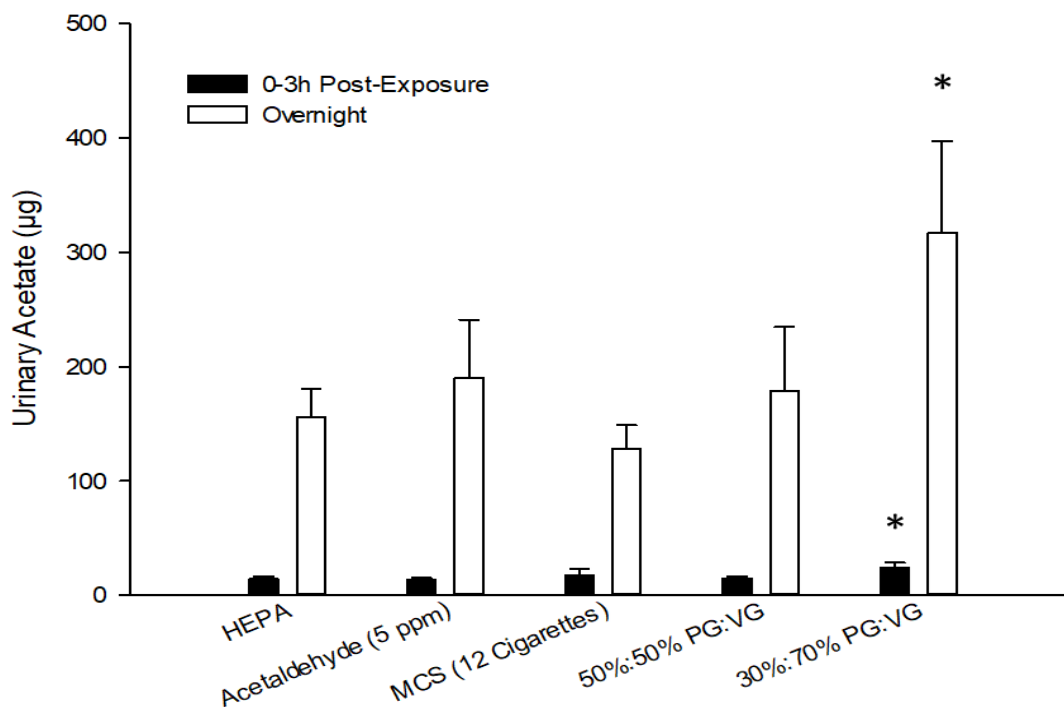


Fig. 5.7. Urinary acetate levels in acetaldehyde- or tobacco product-exposed male mice. Male mice were exposed (6h) to acetaldehyde (5 ppm), mainstream cigarette smoke (MCS; 50% smoke of 12 cigarettes), or propylene glycol:vegetable glycerin (PG:VG; 50%:50% or 30%:70%, 6h) after which urine was collected at 0–1, 1–2, 2–3, and 3–16h (overnight) and acetate quantified. Values are mean \pm SE (n=4-12 mice per group). *, $p < 0.05$ compared with HEPA.

and body weights (or ratios), CBC, or plasma markers (Tables 5.5 & 5.6). For example, exposure to 5 ppm AA caused a modest decrease in final body weight (-4%; $p=0.09$) but a slight increase in the heart/body weight ratio (+8%; $p=0.02$) (Table 5.5), and although low plasma volumes limited many other measures of systemic toxicity, AA-exposed mice showed a modest decrease in cholesterol (-16%; $p=0.05$) compared with their air-exposed control counterparts (Table 5.6).

8. Two-Week Exposure to Acetaldehyde and Circulating Angiogenic Cells

Despite the minimal systemic effects noted in AA-exposed male mice (5 ppm; 2 weeks), AA induced a modest increase in the level of Sca-1⁺ cells (+58%; $p=0.06$), although CACs were not significantly changed (Fig. 5.8A & B).

9. Systemic Toxicity of Acute Exposure to Inhaled Acetaldehyde

Although two weeks of exposure to AA (5 ppm) has minimal systemic toxicity (decreased body weight, decreased cholesterol, increased neutrophils), we performed acute (4d) AA exposures to test whether acute CR exposure would induce rapid toxicity that was otherwise compensated for in a longer setting. Male mice acutely exposed to AA (5 ppm) showed an increased body weight (+11%; $p=0.01$), a modestly increased heart/body weight ratio (+9%; $p=0.06$), and a modestly decreased lung/body weight ratio (-6%; $p=0.06$) (Table 5.5). Plasma levels of cholesterol, HDL, LDL, and albumin were unchanged with acute exposure, while levels of total protein were modestly decreased (-4%; $p=0.07$) (Table 5.5). Acute (4d) exposure to AA (5 ppm) induced a number of hematological changes, decreasing red blood count, hemoglobin, and hematocrit levels (-14%, $p=0.01$; -16%, $p=0.05$; and -12%, $p=0.03$, respectively), while increasing mean platelet volume (+6%; $p=0.04$) (Table 5.6). No changes in body weight, organ/body weight ratios, or CBC were

Table 5.5. Systemic parameters of male C57BL/6 mice acutely exposed to either air or acetaldehyde (AA; 5 ppm).

| Variable | Exposure | | |
|------------------------------|--------------|--------------|---------------|
| | Air Control | AA (5 ppm) | |
| | | 4 days | 2 weeks |
| Final BWT (g) | 28 ± 0 | 31 ± 1* | 27 ± 1# |
| Heart/BWT (mg/g) | 4.8 ± 0 | 5.3 ± 0.2# | 5.2 ± 0.2* |
| Lung/BWT (mg/g) | 5.5 ± 0 | 5.2 ± 0.1# | 5.7 ± 0.2 |
| Liver/BWT (mg/g) | 40.0 ± 1 | 38.4 ± 1.3 | 41.9 ± 1.1 |
| Kidney/BWT (mg/g) | 12.9 ± 1 | 13.2 ± 0.5 | 12.7 ± 0.3 |
| Plasma Measurements | | | |
| Cholesterol (mg/dL) | 65.05 ± 3.46 | 72.37 ± 4.30 | 54.84 ± 3.35* |
| HDL (mg/dL) | 41.68 ± 4.60 | 48.86 ± 4.24 | 35.24 ± 1.06 |
| LDL (mg/dL) | 5.49 ± 0.91 | 5.71 ± 1.07 | N/A |
| Triglycerides (mg/dL) | 49.76 ± 1.71 | N/A | 52.19 ± 2.07 |
| Albumin (g/dL) | 2.94 ± 0.05 | 2.87 ± 0.04 | 2.91 ± 0.06 |
| Total Protein (g/dL) | 4.98 ± 0.08 | 4.76 ± 0.06# | 4.85 ± 0.12 |

Values = mean ± SE (n=5-10 mice per group); Abbr.: **AA**, acetaldehyde; **BWT**, body weight; **HDL**, high-density lipoprotein; **LDL**, low-density lipoprotein; *, p<0.05 compared with air control; #, 0.05≤p≤0.10 compared with air control.

Table 5.6. Hematological measures of male C57BL/6 mice acutely exposed to either air or acetaldehyde (AA; 5 ppm).

| Hematological Measurements | Exposure | | |
|--|-------------|--------------|--------------|
| | Air Control | AA (5 ppm) | |
| | | 4 days | 2 weeks |
| White Blood Cell (K/uL) | 1.10 ± 0.15 | 1.24 ± 0.19 | 1.29 ± 0.18 |
| Neutrophils (K/uL) | 0.32 ± 0.05 | 0.45 ± 0.19 | 0.61 ± 0.12* |
| Lymphocytes (K/uL) | 0.74 ± 0.11 | 0.73 ± 0.10 | 0.66 ± 0.06 |
| Monocytes (K/uL) | 0.04 ± 0 | 0.05 ± 0.01 | 0.03 ± 0.01 |
| Eosinophils (K/uL) | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Basophils (K/uL) | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Red Blood Cell (M/uL) | 8.76 ± 0.12 | 7.52 ± 0.53* | 8.89 ± 0.12 |
| Hemoglobin (g/dL) | 12.5 ± 0.3 | 10.5 ± 0.8* | 13.2 ± 0.1 |
| Hematocrit (%) | 35.6 ± 0.4 | 31.5 ± 2.2* | 35.9 ± 0.4 |
| Mean Corpuscular Volume (fL) | 40.7 ± 0.3 | 41.8 ± 0.2 | 40.4 ± 0.3 |
| Mean Corpuscular Hemoglobin (pg) | 14.2 ± 0.2 | 13.9 ± 0.2 | 14.8 ± 0.1 |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 35.0 ± 0.8 | 33.1 ± 0.4 | 36.6 ± 0.3 |
| Red Cell Distribution Width (%) | 18.3 ± 0.3 | 18.3 ± 0.4 | 19.0 ± 0.5 |
| Platelets (K/uL) | 731 ± 35 | 782 ± 81 | 716 ± 45 |
| Mean Platelet Volume (fL) | 4.4 ± 0.1 | 4.7 ± 0.1* | 4.3 ± 0.1 |

Values = mean ± SE (n=5-10 mice per group); Abbr.: AA, acetaldehyde; *, p<0.05 compared with air control; #, 0.05≤p≤0.10 compared with air control.

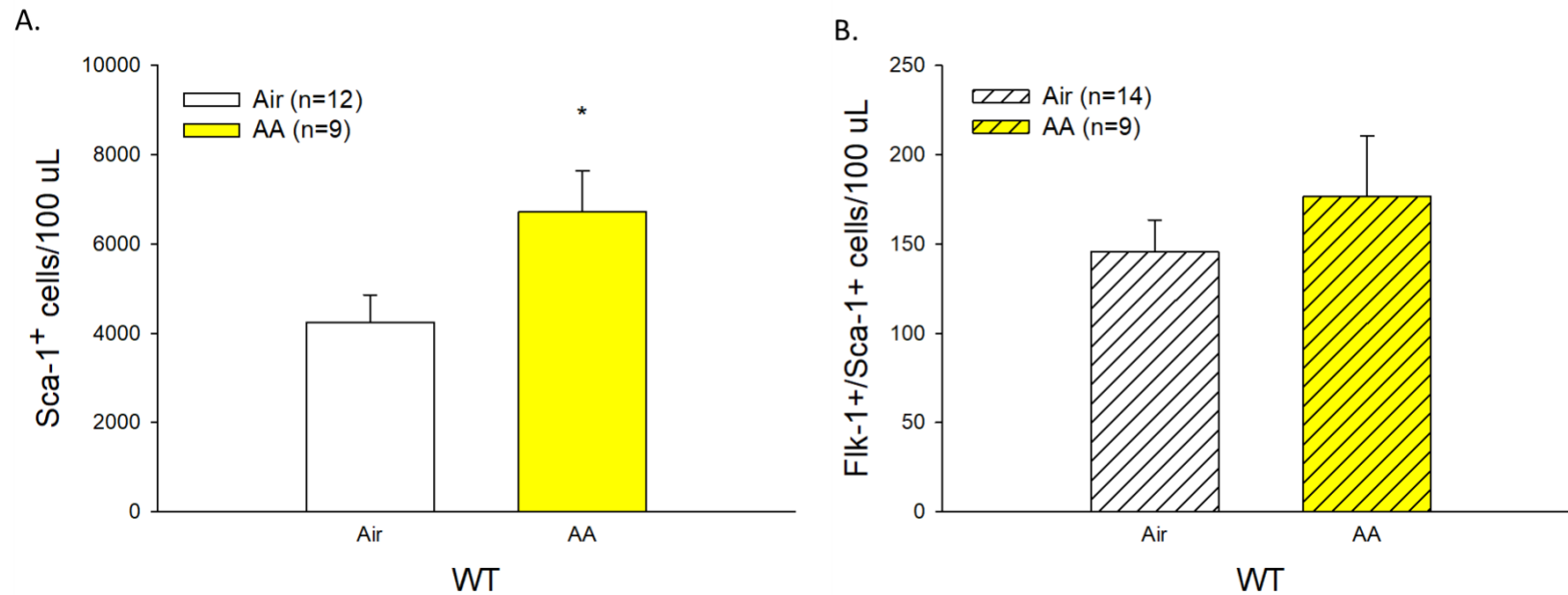


Fig. 5.8. Effects of two-week acetaldehyde (AA; 5 ppm) exposure on circulating stem cells in male mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in male C57BL/6J (wild type, WT) male mice exposed to HEPA-filtered air or acetaldehyde (5 ppm, 6h/d) for two weeks. After two weeks of exposure to AA, the levels of circulating Sca-1⁺ cells were significantly increased in WT mice (**A**), however, no change in the level of CACs (Flk-1⁺/Sca-1⁺ cells) was detected relative to air-exposed control mice (**B**). Values = mean \pm SE (n=9-14 mice per group). *, p<0.05 compared with air control.

observed in female mice exposed for 4 days to 5 ppm AA (Table 5.7); low plasma volumes prevented additional measures of systemic toxicity.

10. Acute Exposure to Acetaldehyde and Circulating Angiogenic Cells

Neither male nor female mice acutely exposed to AA (5 ppm; 4d) showed changes in the levels of CACs or Sca-1⁺ cells (Figs. 5.9A & B, 5.10A & B).

D. Discussion

Exposures to FR and AA have been shown to have negative cardiovascular effects, and they are known to be significant toxins in MCS (96). Although exposure to these saturated aldehydes is fairly ubiquitous, exposure to MCS or secondhand smoke can significantly increase the level of exposure (117). Likewise, new electronic tobacco products including e-cigs, which have become increasingly popular among adolescents (182, 183), have been shown to produce high levels of FR and AA (96, 180), potentially subjecting a new segment of the population to acute, high-level exposures of these compounds. There are still gaps in our understanding of how saturated aldehydes such as FR and AA contribute to the endothelium dysfunction and subsequent CVD linked to MCS and e- cig aerosol exposures. The goal of the current studies was to measure biomarkers of systemic and vascular injury in mice exposed to FR or AA. Furthermore, these studies explored the dependence of concentration, duration, and sex on these outcomes.

Measures of formate, the urinary metabolite of FR, showed that FR generation may differ significantly between tobacco products. Levels of formate in MCS-exposed mice were significantly higher than levels after exposure to 5 ppm FR. Levels of formate differed even between different compositions of PG:VG, with 50%:50% PG:VG, the composition used in blu Plus+ e-cigs, generating higher levels of FR than 30%:70%

Table 5.7. Systemic changes in female C57BL/6 mice acutely exposed to either air or acetaldehyde (AA; 5 ppm).

| Variable | Exposure | |
|---|-------------|-------------|
| | Air Control | AA (5 ppm) |
| Final BWT (g) | 21 ± 0 | 23 ± 1 |
| Heart/BWT (mg/g) | 5.0 ± 0.2 | 5.0 ± 0.2 |
| Lung/BWT (mg/g) | 7.0 ± 0.2 | 6.8 ± 0.2 |
| Liver/BWT (mg/g) | 44.8 ± 0.9 | 46.1 ± 1.8 |
| Kidney/BWT (mg/g) | 12.6 ± 0.4 | 13.0 ± 0.3 |
| Hematological Measurements | | |
| White Blood Cell (K/uL) | 1.60 ± 0.34 | 1.15 ± 0.17 |
| Neutrophils (K/uL) | 0.45 ± 0.14 | 0.28 ± 0.03 |
| Lymphocytes (K/uL) | 1.10 ± 0.19 | 0.82 ± 0.14 |
| Monocytes (K/uL) | 0.04 ± 0.01 | 0.04 ± 0.01 |
| Eosinophils (K/uL) | 0 ± 0 | 0 ± 0 |
| Basophils (K/uL) | 0 ± 0 | 0 ± 0 |
| Red Blood Cell (M/uL) | 9.23 ± 0.05 | 9.32 ± 0.11 |
| Hemoglobin (g/dL) | 11.9 ± 0.1 | 11.7 ± 0.2 |
| Hematocrit (%) | 38.5 ± 0.3 | 38.1 ± 0.5 |
| Mean Corpuscular Volume (fL) | 41.7 ± 0.3 | 40.9 ± 0.7 |
| Mean Corpuscular Hemoglobin (pg) | 13.0 ± 0.1 | 12.5 ± 0.3 |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 31.0 ± 0.3 | 30.6 ± 0.4 |
| Red Cell Distribution Width (%) | 17.7 ± 0.2 | 17.6 ± 0.1 |
| Platelets (K/uL) | 695 ± 36 | 680 ± 22 |
| Mean Platelet Volume (fL) | 4.4 ± 0.1 | 4.4 ± 0.1 |

Values = mean ± SE (n=5-6 mice per group); Abbr.: **AA**, acetaldehyde; **BWT**, body weight.

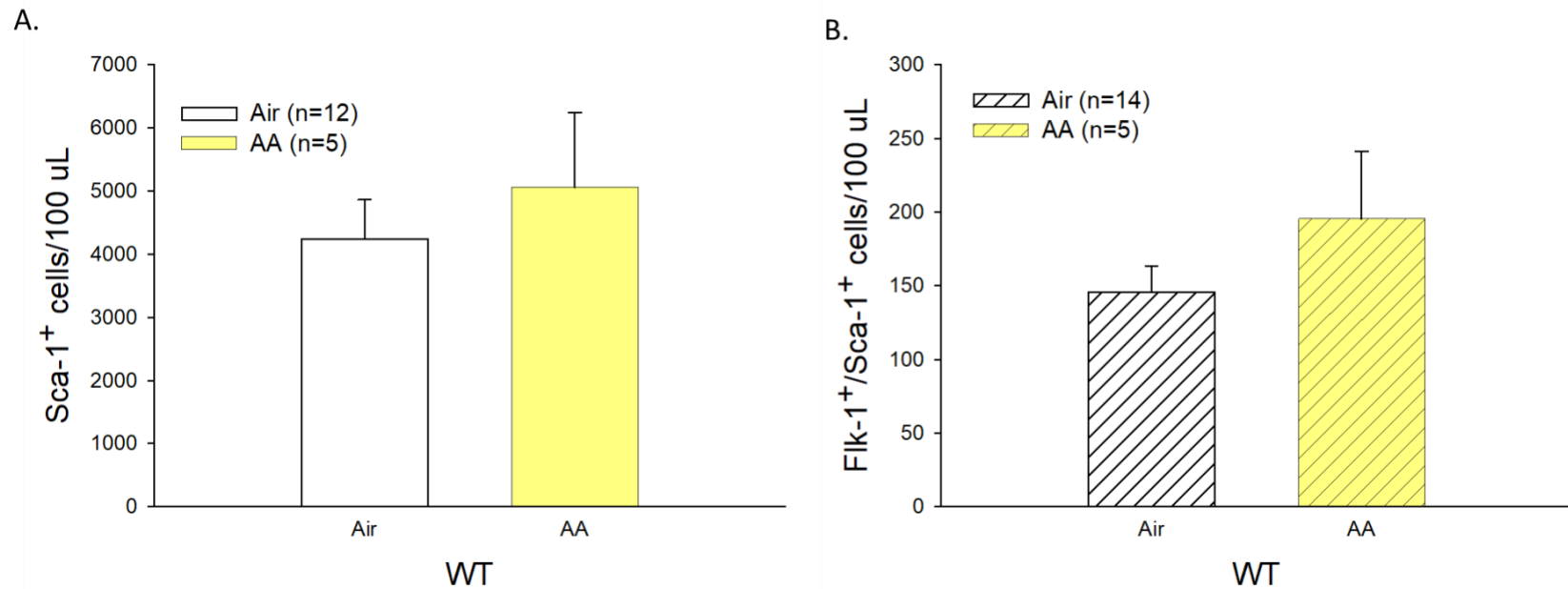


Fig. 5.9. Effects of acute acetaldehyde (AA; 5 ppm) exposure on circulating stem cells in male mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in male C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or acetaldehyde (5 ppm, 6h/d) for 4 days. Levels of circulating Sca-1⁺ cells (**A**) and CACs (Flk-1⁺/Sca-1⁺ cells; **B**) were unchanged in AA-exposed mice relative to air control mice. Values = mean ± SE (n=5-14 mice per group).

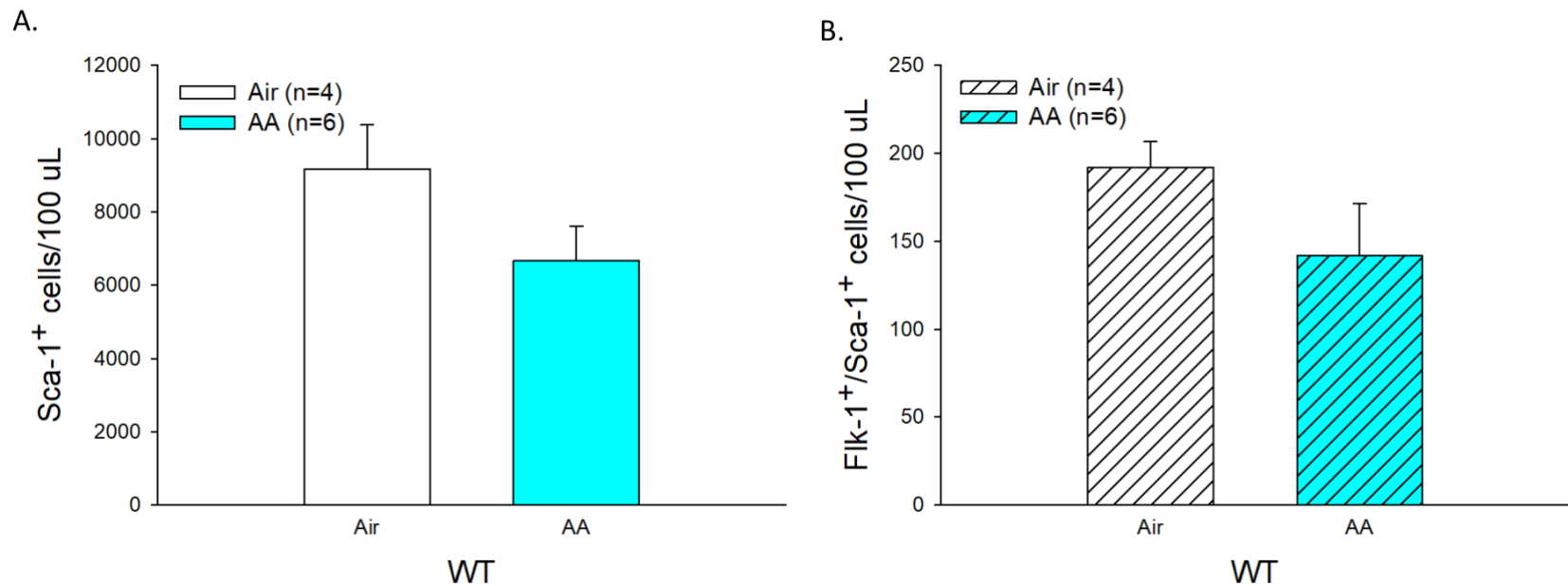


Fig. 5.10. Effects of acute acetaldehyde (AA; 5 ppm) exposure on circulating stem cells in female mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in female C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or acetaldehyde (5 ppm, 6h/d) for 4 days. Levels of circulating Sca-1⁺ cells (**A**) and CACs (Flk-1⁺/Sca-1⁺ cells; **B**) were unchanged in AA-exposed mice relative to air control mice. Values = mean ± SE (n=7-13 mice per group).

PG:VG, the composition used in JUUL devices, although this was not a significant difference. As formate measured in MCS-exposed mice was not significantly different than measured in PG:VG-exposed mice, the current data would suggest that e-cigs do not generate more FR than traditional cigarettes. Additionally, the data suggest that, as there was no significant increase in formate with an increased level of VG in the tested PG:VG ratios, VG is not the primary producer of FR in e-cigs. Previous data, however, have shown that the generation of FR does in fact differ between MCS and e-cigs. Ogunwale et al. (96) report significantly higher levels of FR generation in MCS than in aerosol from a number of different blu cartridges, although levels of FR similar to levels in MCS were measured in a handful of different e-cig liquids outside of the blu brand. As our study only exposed mice to 50% of smoke from 12 KY 3R4F cigarettes, it may be that the amount of FR generated was not sufficient to show a significant increase over levels from PG:VG exposure despite showing a significant increase compared with baseline. Additional studies exposing mice to 100% of the smoke from the same number of cigarettes may show results similar to those found in the literature. Furthermore, measures of aldehyde generation in PG- and VG-alone showed higher levels of FR per puff with VG-alone compared with PG-alone (168). These differences may indicate that PG and VG together are capable of generating FR at different concentrations than generated by the individual humectants.

Measures of urinary acetate, the urinary metabolite of AA, also differed between MCS and PG:VG exposures, with levels measured in MCS-exposed mice similar to levels in 50%:50% PG:VG-exposed mice but lower than those in 30%:70% PG:VG-exposed mice. This again seems to contradict what has previously been reported concerning levels

of AA in MCS and e-cigs (96), but the similar levels measured in 50%:50% and 30%:70% PG:VG-exposed mice is comparable to the similar levels of acetate measured after PG- and VG-alone exposures (168). Exposure to 5 ppm AA produced urinary acetate levels similar to those measured in the 50%:50% PG:VG-exposed mice, likely indicating that the level of AA generated by the equivalent blu Plus+ e-cig devices is around 5 ppm.

Studies have shown that additional factors such as the voltage of the e-cig device can influence aldehyde generation (96, 208). Additionally, flavored e-cig liquids have been shown to produce high levels of both FR and AA (213, 435), and as the use of ENDS has grown increasingly customizable (436), accurate measurements of aldehyde generation—as well as generation of other harmful constituents—have become more difficult. This makes research on the generation of aldehydes by individual components of e-cigs even more imperative, as a baseline understanding of potential product harm must be established in order to understand how the more complex web of factors contributes to e-cig-related health effects. As research into e-cigs and their health effects is still a fairly new field, it is not unexpected that studies may produce varying results, especially as different products, flavors, and humectants may be used for testing. This emphasizes the complexity of e-cigs as well as the need for more research. Nonetheless, our studies show that the concentrations used for our aldehyde-alone exposures produce urinary metabolites at similar levels as produced in MCS- and PG:VG-exposed mice, justifying the use of these concentrations as relevant real-world exposure levels.

Overall, exposures to FR seemed to produce little to moderate systemic effects. Although some significant changes were seen in response to acute exposure to FR at 1, 2, and 5 ppm, these changes are unlikely to be physiologically significant, with changed

measurements typically still within the normal physiological range for specific biomarkers. However, two weeks of exposure to FR (5 ppm) produced significant changes in hematological measures; two-week-exposed male mice showed significant decreases in leukocytes and in platelets, changes which have been previously reported (120, 121). Interestingly, after two weeks, we saw an increase in red blood cell count rather than a decrease as reported in the literature (120), but a significant decrease in red blood cells was seen after four days of exposure at the same level (5 ppm). These results could indicate that FR causes a decrease in red blood cells early on but that homeostatic mechanisms were able to compensate after a longer exposure duration. A similar increase was noted in rats exposed to high-level FR (437), although the authors note that the increased levels were not outside the expected range for that strain of rat and were therefore uncertain of its clinical relevance. Likewise, our values remain within the normal range for this strain of mice, so while the values may not indicate physiological significance, this pattern may point towards changes in red blood cell production that could contribute to cardiovascular injury or disease development. Hemoglobin showed a similar pattern of change between four days and two weeks of exposure at 5 ppm FR.

Biomarkers of vascular injury showed no change after four days of FR (2 ppm), but CACs (Flk-1+/Sca-1+ cells) were significantly increased after four days of FR at a higher concentration (5 ppm), and there was also a trend towards increased levels of Sca-1+ cells. After two weeks of exposure (5 ppm), however, both cell types were decreased, which may indicate that there was significant injury early on which necessitated increased levels of CACs for repair but that these increased levels could not be maintained to repair consistent vascular injury occurring over a longer exposure period. CACs play a role in maintaining

proper endothelium function (62, 63), and decreased levels of these cells can allow for the development of endothelium dysfunction (66, 70). Exposure to other aldehydes such as acrolein (71, 74, 151) as well as MCS itself (72, 318) have also been shown to decrease the levels and function of CACs and contribute to endothelium dysfunction, although the shorter time frame in which these effects are produced likely indicates that acrolein is more toxic for these cells than FR. Wheat et al. (74) have shown that acrolein decreases levels of CACs in the blood through the inhibition of vascular endothelial growth factor-induced mobilization of these cells from the bone marrow; future studies of FR exposed should examine levels of CACs in the bone marrow to determine whether this aldehyde's effect on CACs is the result of this mechanism. Nonetheless, the decrease in CACs noted after two weeks of exposure may indicate that FR is capable of inducing endothelium dysfunction, which has been previously suggested (438). However, *ex vivo* studies in blood vessels did not observe evidence of endothelium dysfunction even in response to high levels of FR (127). This discrepancy could indicate that the effect of FR on endothelium function varies between intact animals and *ex vivo* vessels, but more studies are needed to better understand this relationship.

Exposure to AA (5 ppm, 4 days and 2 weeks) showed minimal systemic and hematological effects. Unlike FR, AA-exposed mice showed no significant changes in overall leukocyte counts, and although mice exposed to AA for four days showed decreases in red blood cell count, hemoglobin, and hematocrit, these changes were not seen after two weeks of exposure. Two weeks of exposure did cause a significant increase in Sca-1+ cells, but CACs remained unchanged. One study has found a link between AA and the potential development of endothelium dysfunction (439), but this hypothesis is still largely

unexplored. Although our findings do not suggest that AA is linked to the development of endothelium dysfunction, studies with a larger number of mice and for longer durations would allow for a more definitive clarification of the relationship between AA and endothelium dysfunction.

We also sought to determine whether saturated aldehyde-induced effects on systemic and hematological biomarkers and CACs are sex-dependent. Like their male counterparts, female WT mice exposed to FR (2 or 5 ppm; 4d) or AA (5 ppm; 4d) showed minimal cardiovascular effects. The few significant changes noted in response to FR exposure (2 or 5 ppm) are not likely to be physiologically significant, and no changes were noted in AA-exposed mice. Likewise, neither FR nor AA exposure in female mice induced changes in CACs. The difference in outcomes between male and female mice exposed to FR or AA at the same concentration and for similar durations may indicate that the aldehyde-induced cardiovascular changes are sex-dependent, particularly the FR-induced effects in CACs, although more robust studies are needed to truly make this determination. Although a small number of studies have reported sex differences in response to other aldehydes (269, 440), sex-related differences in acetaldehyde exposures have mainly been explored in relation to AA ingestion rather than inhalation (441-444), and of the historical studies examining long-term effects of formaldehyde exposure (445-449), only Maronpot et al. (445) report significant differences between males and females. Our studies therefore represent novel observations regarding the potential sex-dependence of full-body saturated aldehyde inhalation exposure on cardiovascular effects in mice, especially in regard to differences in biomarkers of vascular injury in response to FR exposure.

In summary, the studies presented in this chapter have demonstrated the importance

of research into the saturated aldehydes FR and AA as potential contributors of systemic and vascular injury in response to inhalation exposure. Levels of these constituents were measured at levels comparable to those generated in response to exposure from MCS and varying compositions of PG:VG, indicating their real-world relevance in determining the health effects of e-cig use. Two weeks of exposure to FR (5 ppm) in particular induced significant systemic changes that confirm the results of previous studies and implicate FR as a promoter of CVD. This exposure also induced changes in CACs that suggest FR may play a role in the development of endothelium dysfunction *in vivo*. Furthermore, the CAC effects induced by FR seem to be sex-dependent, although additional studies are needed to confirm this finding. As smoking conventional cigarettes remains a global health threat and as the number of e-cig users continues to increase, these studies provide novel insight into the role of saturated aldehydes in the development of CVD and suggest differences between male and females that could play an important role in the therapeutic treatment of FR exposure.

CHAPTER VI

SYSTEMIC, HEMATOLOGICAL, AND ENDOTHELIUM-RELATED EFFECTS OF ACROLEIN AND CROTONALDEHYDE IN MICE

A. Introduction

The unsaturated aldehydes acrolein and crotonaldehyde (CR) are classified as eye, nose, skin, and respiratory irritants, with inhalation of even low levels capable of producing extreme respiratory distress (94, 155). Exposure to both acrolein and CR can occur naturally through foods and drinks (94), and each is produced endogenously in small amounts (94, 155-157). Most exposure to these compounds, however, occurs via anthropogenic sources, including vehicular exhaust, industrial emissions, and smoke from fires (94, 155-157), with a large portion of exposure related to tobacco smoke (90, 114, 117, 147, 156, 159). Acrolein in particular plays a significant role in mainstream cigarette smoke (MCS) and its related health effects; Stabbert et al. (149) have shown that the majority of the cytotoxicity related to the gas vapor constituents of MCS is due to acrolein, and Hausmann (145) reports that almost 90% of the non-cancer health risk (cardiovascular and pulmonary disease) associated with smoking is likely due to acrolein.

Much epidemiological and experimental evidence exists linking acrolein with increased cardiovascular disease (CVD) risk. Both acute and chronic inhalation exposures

to acrolein have been shown to suppress levels of circulating angiogenic cells (CACs) (74, 151), enhance thrombotic markers (450), and initiate endothelium dysfunction in susceptible mice (150), while ingestion promotes atherosclerosis in mice (153). Moreover, urinary levels of 3-hydroxypropylmercapturic acid (3HPMA), the primary urinary metabolite of acrolein, are associated with increased CVD risk, decreased levels of CACs, and increased platelet-leukocyte aggregation in humans (71). By comparison, inhalation exposure to 1,3-butadiene, a parent source of CR, accelerates arteriosclerotic lesion development in cockerels (167), and is linked with an increased risk of atherosclerosis in African-American factory workers (166). Likewise, African-American men who worked in a 1,3-butadiene production plant for at least 6 months had increases in standardized mortality ratio for arteriosclerotic heart disease (165). Furthermore, although CR has been shown to induce oxidative stress in cells (163, 164) and to cause cardiomyocyte dysfunction (164), CR inhalation studies with measures of cardiovascular injury have not been performed. Thus, the goal of these studies was to address these gaps in knowledge regarding the potential cardiovascular toxicity of CR.

B. Experimental Procedures

1. Exposures

Animals were exposed to constituents via full-body inhalation as described in Chapter II, Sections B.2. Upon completion of the exposure, mice were euthanized and tissues collected as described in Chapter II, Section B.5.

2. Urine Collection

Urine samples were collected post-exposure as described in Chapter II, Section B.3.

3. Urine Metabolite Quantification

Collected urine was used to quantify levels of volatile organic compound (VOC) and tobacco alkaloid metabolites using analytical methods as described in Chapter II, Section B.4.

4. Complete Blood Cell Count (CBC)

A sample of whole blood was collected after the final day of exposure for a complete blood cell count (CBC) as described in Chapter II, Section B.6.

5. Plasma Biomarkers Quantification

Plasma was prepared from each sample for an analysis of a number of biomarkers as described in Chapter II, Section B.7.

6. Circulating Angiogenic Cell (CAC) Quantification

Flow cytometry was used to analyze the number of CACs in each sample as described in Chapter II, Section B.8.

7. Leukocyte Subpopulation Quantification

Flow cytometry was used to analyze the leukocyte subpopulations in each sample as described in Chapter II, Section B.9.

8. Statistical Analyses

Statistical analyses were performed as described in Chapter II, Section B.10.

C. Results

1. Urinary Acrolein Metabolite in MCS-, PG:VG-, and Acrolein-Exposed Mice

The urinary metabolite of acrolein, 3HPMA, has been positively associated with increased CVD risk in smokers (71) and has been shown to be a useful biomarker of exposure to acrolein from various sources (147, 151). However, the use of 3HPMA as a

biomarker of e-cig exposure has not been studied. To better understand how acrolein-alone exposure related to e-cig exposure from known ratios of PG:VG, we measured urinary 3HPMA by UPLC-MS/MS as a biomarker of acrolein exposure. For this, male mice were exposed to acrolein (1 ppm, 6h), MCS (50% of smoke of 12 KY Reference 3R4F cigarettes, 6h), or PG:VG (50%:50% or 30%:70%, 6h), and urine was collected immediately after the exposure sessions. Acrolein alone exposure ($p=0.02$) as well as exposure to MCS ($p=0.005$) and both 50%:50% ($p<0.001$) and 30%:70% PG:VG ($p=0.02$) caused a significant increase in levels of 3HPMA in the first three hours post exposure compared with baseline (Fig. 6.1). Levels of 3HPMA in acrolein-exposed mice were similar to levels in MCS-exposed and 30%:70% PG:VG-exposed mice, but mice exposed to 50%:50% PG:VG showed significantly higher levels compared to acrolein-alone mice ($p=0.01$) (Fig. 6.1). Comparison of 3HPMA levels in PG:VG-exposed mice showed that mice exposed to 50%:50% PG:VG also had a significantly higher level of 3HPMA compared with mice exposed to 30%:70% PG:VG ($p=0.02$) (Fig. 6.1).

2. Systemic Toxicity of Acute Exposure to Inhaled Acrolein

The unsaturated aldehyde acrolein is found in high levels in MCS (90, 114, 117, 147) and is suggested to be one of the most significant factors in MCS-induced cardiovascular toxicity (145, 149). Environmental exposure to acrolein has been linked to an increased CVD risk score, in part through decreased levels of select CAC populations (71), and direct inhalation exposure in mice has also caused the suppression of CACs (74, 151) as well as the related development of endothelium dysfunction (150). Chronic inhalation exposure (1 ppm) showed significant decreases in white blood cells count driven by decreases in neutrophils, lymphocytes, and monocytes (151). Because of these changes

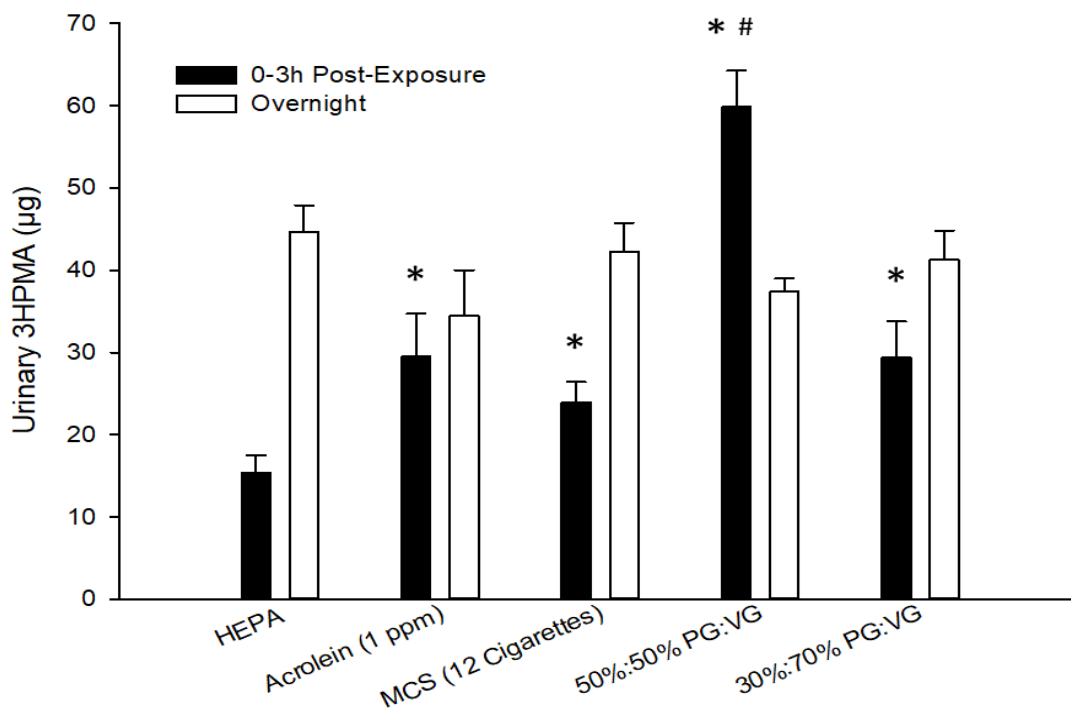


Fig. 6.1. Urinary 3HPMA levels in acrolein- or tobacco product-exposed male mice.

Male mice were exposed (6h) to mainstream cigarette smoke (MCS; 50% smoke of 12 KY Reference 3R4F cigarettes) or propylene glycol:vegetable glycerin (PG:VG; 50%:50% or 30%:70%, 6h) after which urine was collected at 0–1, 1–2, 2–3, and 3–16h (overnight) and 3-hydroxypropylmercapturic acid (3HPMA) quantified. Values are mean \pm SE (n=4-12 mice per group). *, p<0.05 compared with HEPA; #, p<0.05 compared with acrolein.

noted in humans and in chronically-exposed mice, we performed an acute acrolein exposure to better understand the temporal relationship between these factors. Male mice exposed to acrolein (1 ppm) for 4 days (6h/d) had a greater change in overall body weight compared with air-exposed control mice (-3 g versus -1 g; $p=0.004$), and the liver/body weight ratio was significantly decreased (-10%; $p=0.007$) (Table 6.1). Exposure had considerable effects on CBC. Exposed mice showed a significant decrease in leukocytes (-50%; $p=0.02$), driven by decreases in neutrophils (-57%; $p=0.02$), lymphocytes (-46%; $p=0.06$), and monocytes (-59%; $p=0.03$) (Table 6.2). Acrolein exposure also induced changes in erythrocytic parameters; red blood cell levels (+6%; $p=0.05$) and hemoglobin (+5%; $p=0.06$) were modestly increased, while hematocrit was significantly increased (+6%; $p=0.04$) (Table 6.2). Measures of systemic biomarkers showed no changes in liver (ALT, AST), muscle (CK) or LDH enzymes or creatinine but did show a modestly significant decrease in triglycerides (-22%; $p=0.06$) (Table 6.1).

3. Acute Exposure to Acrolein and Circulating Angiogenic Cells

Male mice acutely exposed to acrolein (1 ppm; 4d) showed no changes in the levels of CACs or Sca-1⁺ cells (Fig. 6.2A & B).

4. Urinary Crotonaldehyde Metabolite in MCS-, PG:VG-, and Crotonaldehyde-Exposed Mice

The primary urinary metabolite of CR, 3-hydroxy-1-methylpropylmercapturic acid (HPMMA), is associated with combusted tobacco, and the number of cigarettes per day is a significant predictor of increased urinary HPMMA (159). To better understand how CR-alone exposure relates to MCS exposure from a known number of cigarettes and to e-cig exposure using a known ratio of PG:VG, we measured urinary HPMMA by UPLC-MS/MS

Table 6.1. Systemic parameters of male C57BL/6 mice acutely exposed to either air or acrolein (1 ppm).

| Variable | Exposure (4 days) | |
|------------------------------|-------------------|------------------|
| | Air Control | Acrolein (1 ppm) |
| Change in BWT (g) | -1 ± 0 | -3 ± 0* |
| Heart/BWT (mg/g) | 4.8 ± 0.1 | 5.0 ± 0.1 |
| Lung/BWT (mg/g) | 5.7 ± 0.1 | 5.6 ± 0.1 |
| Liver/BWT (mg/g) | 46.0 ± 1.1 | 41.3 ± 0.08* |
| Kidney/BWT (mg/g) | 12.8 ± 0.2 | 12.6 ± 0.2 |
| Plasma Measurements | | |
| Cholesterol (mg/dL) | 74.89 ± 3.35 | 70.67 ± 2.6 |
| Triglycerides (mg/dL) | 55.33 ± 4.31 | 43.36 ± 3.36# |
| Albumin (g/dL) | 2.92 ± 0.13 | 2.90 ± 0.23 |
| Total Protein (g/dL) | 3.94 ± 0.06 | 4.47 ± 0.18 |
| AST (U/I) | 65.64 ± 6.70 | 60.94 ± 5.56 |
| CK (U/I) | 144.14 ± 41.37 | 105.87 ± 21.03 |
| LDH (U/I) | 178.37 ± 20.37 | 164.29 ± 21.52 |
| Creatinine (mg/dL) | 0.22 ± 0.01 | 0.23 ± 0.02 |

Values = mean ± SE (n=5-10 mice per group); Abbr.: **AST**, aspartate aminotransferase; **BWT**, body weight; **CK**, creatine kinase; **LDH**, lactate dehydrogenase; #, 0.05 ≤ p ≤ 0.10 compared with air control.

Table 6.2. Hematological measurements of male C57BL/6 mice acutely exposed to either air or acrolein (1 ppm).

| Hematological Measurements | Exposure (4 days) | |
|---|--------------------------|-------------------------|
| | Air Control | Acrolein (1 ppm) |
| White Blood Cell (K/uL) | 1.92 ± 0.34 | 0.96 ± 0.13* |
| Neutrophils (K/uL) | 0.63 ± 0.13 | 0.27 ± 0.04* |
| Lymphocytes (K/uL) | 1.24 ± 0.23 | 0.68 ± 0.09# |
| Monocytes (K/uL) | 0.04 ± 0.01 | 0.02 ± 0.01* |
| Eosinophils (K/uL) | 0 ± 0 | 0 ± 0 |
| Basophils (K/uL) | 0 ± 0 | 0 ± 0 |
| Red Blood Cell (M/uL) | 8.83 ± 0.15 | 9.38 ± 0.16# |
| Hemoglobin (g/dL) | 12.2 ± 0.3 | 12.9 ± 0.2# |
| Hematocrit (%) | 44.0 ± 0.9 | 46.7 ± 0.7* |
| Mean Corpuscular Volume (fL) | 49.8 ± 0.4 | 49.8 ± 0.3 |
| Mean Corpuscular Hemoglobin (pg) | 13.8 ± 0.1 | 13.8 ± 0.1 |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 27.8 ± 0.2 | 27.6 ± 0.1 |
| Red Cell Distribution Width (%) | 17.5 ± 0.2 | 17.1 ± 0.2 |
| Platelets (K/uL) | 575 ± 27 | 585 ± 14 |
| Mean Platelet Volume (fL) | 4.3 ± 0.1 | 4.2 ± 0.1 |

Values = mean ± SE (n=10 mice per group); *, p<0.05 compared with air control; #, 0.05≤p≤0.10 compared with air control.

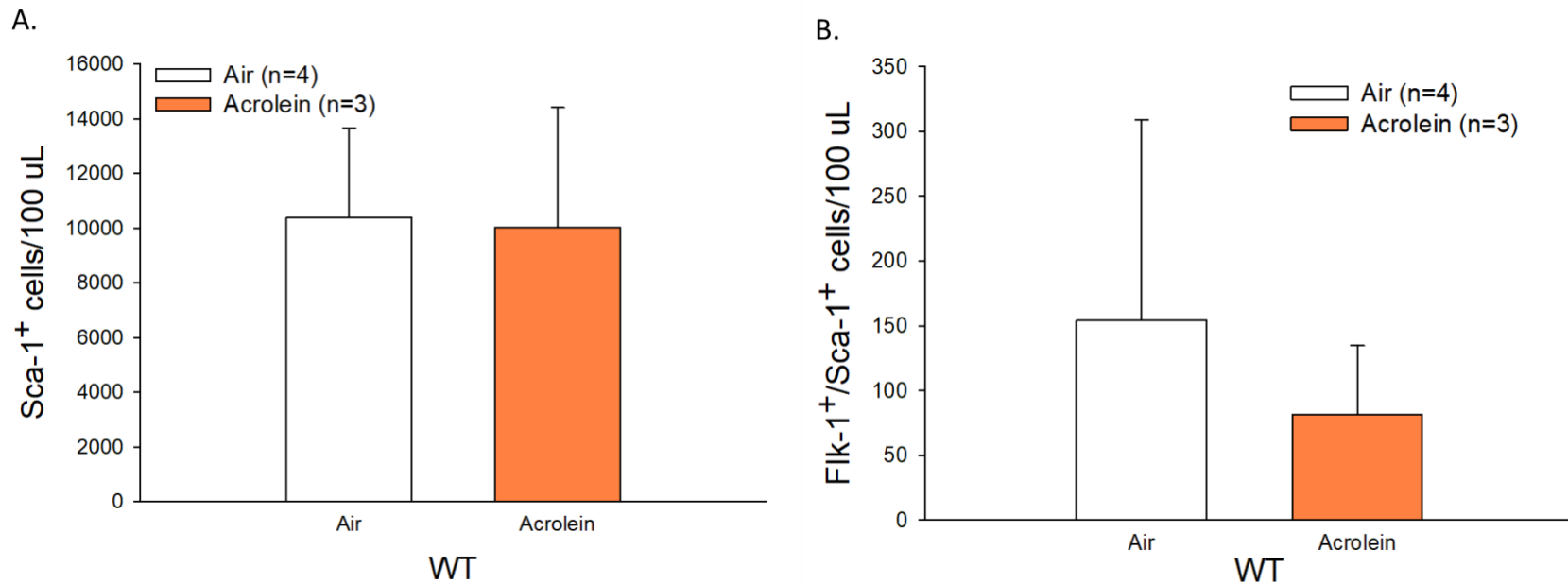


Fig. 6.2. Effects of acute acrolein (1 ppm) exposure on circulating stem cells in male mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in male C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or acrolein (1 ppm, 6h/d) for 4 days. Levels of circulating Sca-1⁺ cells (**A**) and CACs (Flk-1⁺/Sca-1⁺ cells; **B**) were unchanged in acrolein-exposed mice relative to air-exposed control mice. Values = mean ± SE (n=3-4 mice per group).

as a biomarker of CR exposure (168). For this, male mice were exposed to CR (1 ppm, 6h), MCS (50% of smoke of 12 KY Reference 3R4F cigarettes, 6h) or 50%:50% PG:VG (6h), and urine was collected immediately after each exposure session. Urinary HPMMA levels were significantly increased in mice exposed to CR ($p < 0.001$) or MCS ($p < 0.001$) compared with baseline in the first three hours post exposure, while levels in 50%:50% PG:VG-exposed mice remained near baseline (Fig. 6.3). Mice exposed to CR-alone also showed significantly higher levels of urinary HPMMA than mice exposed to MCS ($p = 0.003$) or 50%:50% PG:VG ($p = 0.007$) (Fig. 6.3). Unlike humans, laboratory-housed, naïve mice have modest HPMMA levels in urine, indicating minimal CR exposure via exogenous sources including ambient air or intermediary metabolism (Fig. 6.3). After 11 weeks of exposure to 1 ppm CR exposure, the level of urinary HPMMA in the first three hours post-exposure was approximately 5-times that measured following acute MCS exposure (Fig. 6.3), indicating that MCS exposure was equivalent to 0.2 ppm of continuous CR exposure.

5. Systemic Toxicity of Chronic Exposure to Inhaled Crotonaldehyde

Because chronic exposure to acrolein (12 weeks, 1 ppm) induced significant systemic toxicity in mice (151), we tested whether chronic CR (1 ppm) exposure would elicit similar outcomes. Body weight and organ/body weight ratios and CBCs were not different between the air- and CR- exposed groups (Tables 6.3 & 6.4). Chronic CR also did not affect plasma levels of ALT and AST (liver), CK (striated muscle), or LDH (non-specific cell toxicity) enzymes or creatinine, but did increase levels of plasma total cholesterol (+11%; $p = 0.04$), HDL (+16%; $p = 0.01$), triglycerides (+25%; $p = 0.01$), albumin (+6%; $p = 0.01$), and, modestly, total protein (+3%; $p = 0.08$) compared with the air-exposed

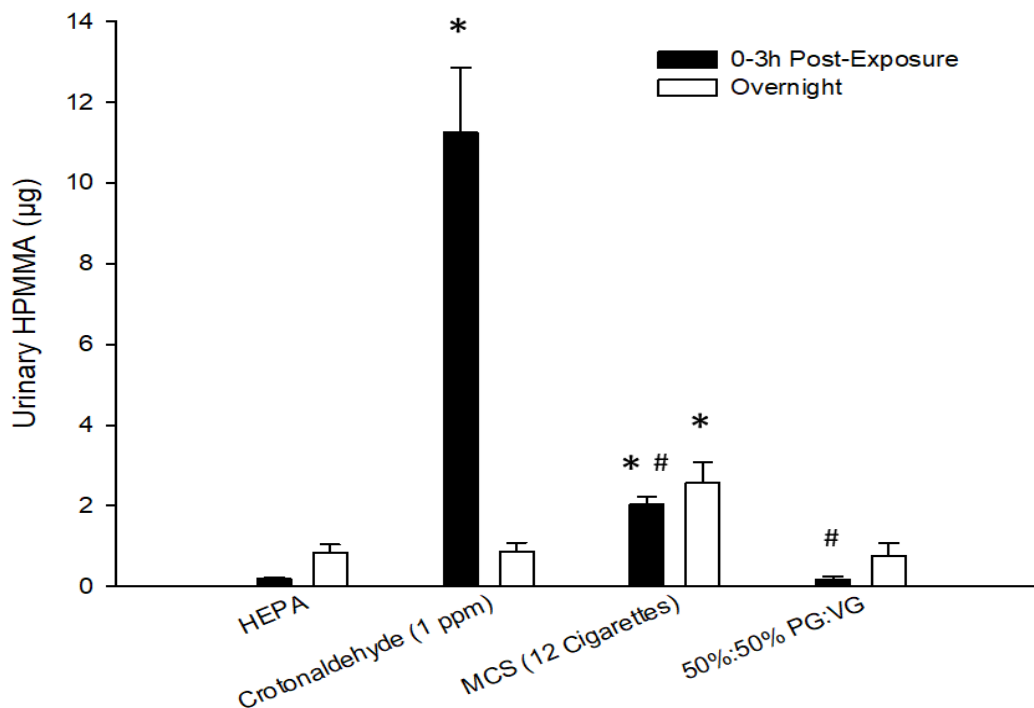


Fig. 6.3. Urinary HPMMA levels in crotonaldehyde- or tobacco product-exposed male mice. Male mice were exposed (6h) to crotonaldehyde (CR; 1 ppm, 11th week of chronic exposure), mainstream cigarette smoke (MCS; 50% smoke of 12 KY Reference 3R4F cigarettes), or propylene glycol:vegetable glycerin (PG:VG; 50%:50% or 30%:70%, 6h) after which urine was collected at 0–1, 1–2, 2–3, and 3–16h (overnight) and 3-hydroxy-1-methylpropylmercapturic acid (HPMMA) quantified. Values are mean \pm SE (n=5-12 mice per group). *, p<0.05 compared with HEPA; #, p<0.05 compared with CR.

Table 6.3. Systemic parameters in male C57BL/6J mice chronically exposed to either air or crotonaldehyde (CR; 1 ppm).

| Variable | Exposure | |
|-------------------------------|----------------|----------------|
| | Air Control | CR (1 ppm) |
| Change in BWT (g) | +5 ± 0 | +4 ± 0 |
| Heart/BWT (mg/g) | 4.5 ± 0.1 | 4.6 ± 0.1 |
| Lung/BWT (mg/g) | 5.4 ± 0.2 | 5.4 ± 0.2 |
| Liver/BWT (mg/g) | 44.3 ± 1.6 | 41.8 ± 1.7 |
| Kidney/BWT (mg/g) | 12.3 ± 0.3 | 13.1 ± 0.5 |
| Pancreas/BWT (mg/g) | 3.9 ± 0.2 | 4.6 ± 0.4 |
| Spleen/BWT (mg/g) | 3.1 ± 0.2 | 3.0 ± 0.2 |
| Epididymal Fat Pad/BWT (mg/g) | 8.8 ± 0.5 | 9.0 ± 0.8 |
| Plasma Measurements | | |
| Cholesterol (mg/dL) | 58.83 ± 3.82 | 69.51 ± 2.10* |
| HDL (mg/dL) | 41.93 ± 4.04 | 53.05 ± 0.94* |
| LDL (mg/dL) | 4.94 ± 1.01 | 3.51 ± 0.33 |
| Triglycerides (mg/dL) | 37.12 ± 2.85 | 56.56 ± 7.31* |
| Albumin (g/dL) | 2.81 ± 0.06 | 2.89 ± 0.07* |
| Total Protein (g/dL) | 4.52 ± 0.08 | 4.67 ± 0.08# |
| ALT (U/I) | 19.37 ± 3.76 | 15.95 ± 1.46 |
| AST (U/I) | 49.43 ± 13.16 | 46.74 ± 4.56 |
| CK (U/I) | 260.92 ± 39.83 | 149.57 ± 44.55 |
| LDH (U/I) | 83.03 ± 8.30 | 83.65 ± 4.80 |
| Creatinine (mg/dL) | 0.25 ± 0.02 | 0.25 ± 0.01 |

Values = mean ± SE (n=9-10 mice per group). **Abbr.:** **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **BWT**, body weight; **CK**, creatine kinase; **CR**, crotonaldehyde; **HDL**, high-density lipoprotein; **LDH**, lactate dehydrogenase; **LDL**, low-density

lipoprotein; *, $p < 0.05$ compared with air control based on Bonferroni's post-test; #, $0.05 \leq p \leq 0.10$ compared with air control based on Bonferroni's post-test.

Table 6.4. Hematological measures of male C57BL/6J mice chronically exposed to either air or crotonaldehyde (CR; 1 ppm).

| Hematological Measurements | Exposure | |
|--|--------------------|--------------------|
| | Air Control | CR (1 ppm) |
| White Blood Cell (K/ μ L) | 1.96 \pm 0.19 | 2.12 \pm 0.18 |
| Neutrophils (K/ μ L) | 0.50 \pm 0.08 | 0.60 \pm 0.07 |
| Lymphocytes (K/ μ L) | 1.41 \pm 0.12 | 1.47 \pm 0.14 |
| Monocytes (K/ μ L) | 0.05 \pm 0.01 | 0.04 \pm 0.01 |
| Eosinophils (K/ μ L) | 0 \pm 0 | 0 \pm 0 |
| Basophils (K/ μ L) | 0 \pm 0 | 0 \pm 0 |
| Red Blood Cell (M/ μ L) | 8.30 \pm 0.15 | 8.32 \pm 0.14 |
| Hemoglobin (g/dL) | 12.1 \pm 0.1 | 12.0 \pm 0.1 |
| Hematocrit (%) | 35.1 \pm 0.7 | 34.8 \pm 0.5 |
| Mean Corpuscular Volume (fL) | 42.3 \pm 0.2 | 41.8 \pm 0.3 |
| Mean Corpuscular Hemoglobin (pg) | 14.6 \pm 0.2 | 14.4 \pm 0.3 |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 34.6 \pm 0.5 | 34.5 \pm 0.5 |
| Red Cell Distribution Width (%) | 18.0 \pm 0.3 | 18.0 \pm 0.2 |
| Platelets (K/ μ L) | 728 \pm 30 | 694 \pm 31 |
| Mean Platelet Volume (fL) | 4.2 \pm 0.0 | 4.3 \pm 0.1 |
| Blood Immune Cells | | |
| NK1.1 ⁺ -Cells (count/ μ L) | 8.22 \pm 1.57 | 7.00 \pm 1.11 |
| CD19 ⁺ B-Cells (count/ μ L) | 266.72 \pm 31.36 | 291.24 \pm 51.23 |
| CD4 ⁺ T-Cells (count/ μ L) | 31.43 \pm 2.91 | 33.79 \pm 3.10 |
| CD8 ⁺ T-Cells (count/ μ L) | 0.85 \pm 0.17 | 1.04 \pm 0.18 |
| CD11b ⁺ Monocytes (count/ μ L) | 25.01 \pm 1.95 | 27.06 \pm 2.65 |
| Ly6C ^{high} Cells (count/ μ L) | 1.2 \pm 0.2 | 1.9 \pm 0.4 |
| Ly6C ^{low} Cells (count/ μ L) | 23.8 \pm 1.9 | 25.2 \pm 2.5 |
| Gr1 ⁺ Granulocytes (count/ μ L) | 69.87 \pm 5.21 | 82.52 \pm 7.51 |

Values = mean \pm SE (n=9-10 mice per group). **Abbr.:** CR, crotonaldehyde.

control group (Table 6.3).

6. Chronic Exposure to Crotonaldehyde and Hematology

Chronic exposures to acrolein (12 weeks, 0.5 and 1 ppm) suppress a number of leukocyte subpopulations (151). Thus, in an attempt to understand any selective hematological effects of CR, we used hematological measures to quantify changes in circulating leukocytes and selective immune subpopulations of mice chronically exposed to CR. CR-exposed mice had no changes in major leukocyte classes, and there were no significant changes in levels of NK1.1⁺-cells, CD19⁺ B-cells, CD4⁺ or CD8⁺ T-cells, Gr1⁺ granulocytes, CD11b⁺ monocytes, or Ly6C^{high} or Ly6C^{low} monocyte sub-populations (Table 6.4).

7. Chronic Exposure to Crotonaldehyde and Circulating Angiogenic Cells

Decreased levels of CACs have been associated with increased CVD risk, and it is thought that CAC levels serve as a predictor of overall cardiovascular health and future cardiovascular events (66). Altered CAC levels are noted in individuals exposed to secondhand smoke (72) and during smoking cessation (318). Previously, we have shown that both (74) acute and chronic (151) exposures of mice to 1 ppm acrolein suppressed CACs by 50%. Despite the changes seen with acrolein, there was no significant change in the level of Sca-1⁺ or CACs (Flk-1⁺/Sca-1⁺ cells) after chronic CR exposure (Fig. 6.4A & B).

8. Systemic Toxicity of Acute Exposure to Inhaled Crotonaldehyde

Although chronic exposure to CR (1 ppm) had specific yet limited systemic toxicity, we performed acute CR exposures to better understand the temporal relationships between these factors. Thus, we tested whether acute CR exposures would induce rapid

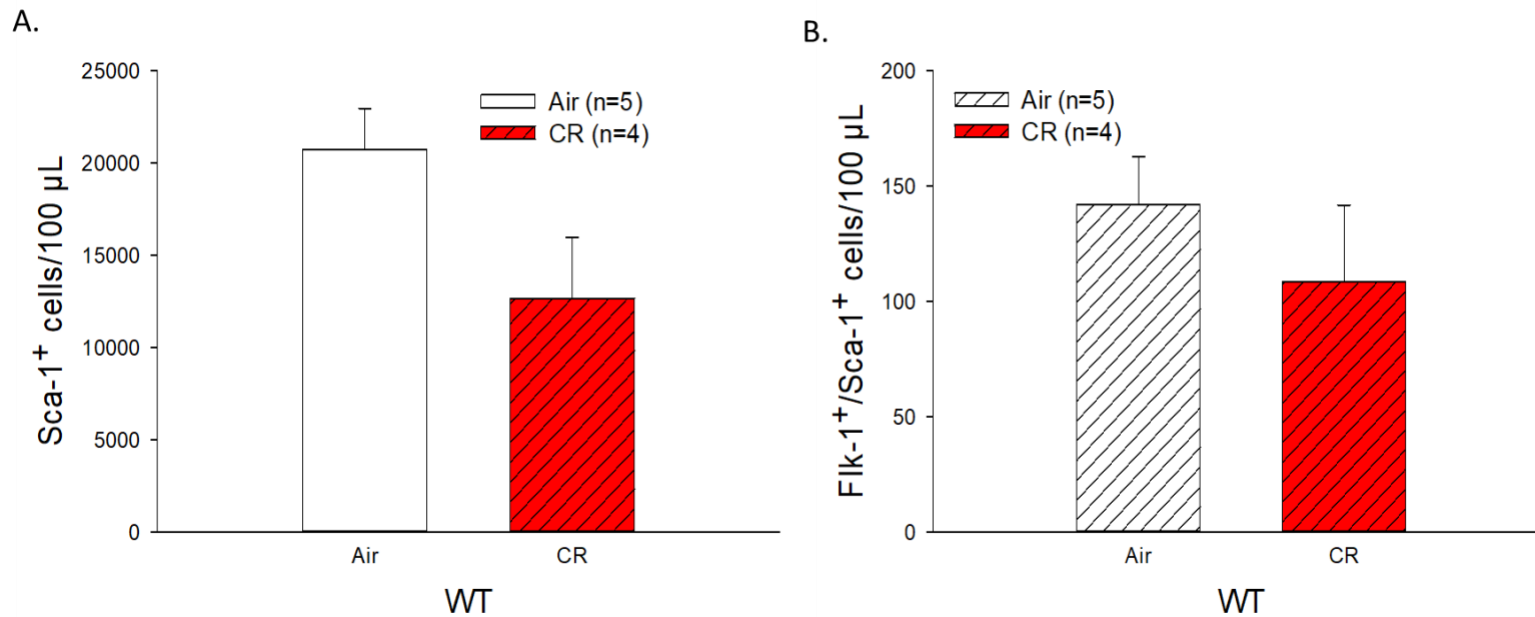


Fig. 6.4. Effects of chronic crotonaldehyde (CR; 1 ppm) exposure on circulating stem cells in male mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in male C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or CR (1 ppm, 6h/d) for 12 weeks. Levels of circulating Sca-1⁺ cells (**A**) and CACs (Flk-1⁺/Sca-1⁺ cells; **B**) were unchanged in CR-exposed mice relative to air control mice. Values = mean ± SE (n=4-5 mice per group).

toxicity that was otherwise compensated for in the chronic setting. Acute exposure of male mice to CR (1 or 3 ppm; 4 days) had limited to modest effects on organ and body weights (or ratios), CBC, and plasma biomarkers. Male mice acutely exposed to 1 ppm CR had no change in overall body weight, yet had an increased level of neutrophils (+60%; $p=0.06$), a decreased mean corpuscular volume (-6%; $p<0.001$), and an increased red blood cell distribution width (+6%; $p=0.02$) (Table 6.5). Plasma levels of ALT, ASK, CK, and LDH enzymes, albumin, and creatinine were unchanged with acute CR exposure in WT male mice, however, levels of plasma cholesterol, LDL, triglycerides and total protein were decreased (-14%, $p=0.002$; -6%, $p=0.02$; -13%, $p=0.02$; -24%, $p=0.002$, respectively) (Table 6.6). Surprisingly, 4 days of CR (3 ppm) exposure in male mice had less of an effect overall on markers of systemic toxicity. For example, there were no changes in levels of ALT, AST, CK, and LDH enzymes, albumin, or creatinine or any changes in hematological biomarkers, with only modest effects on levels of cholesterol (+7%; $p=0.09$) and total protein (+5%; $p=0.07$) (Tables 6.6). Similarly, no changes in body weight, organ/body weight ratios, CBC, or plasma biomarkers were observed in female mice exposed for 4 days to 1 ppm CR (Table 6.7), indicating that male WT mice may be more sensitive to CR than their female counterparts.

9. Acute Exposure to Crotonaldehyde and Circulating Angiogenic Cells

Acute (4d) exposure to 1 ppm CR increased the level of Sca-1⁺ cells (Fig. 6.5A); Sca-1⁺ cells make up the hematopoietic stem cell pool from which CACs are derived. However, there was no change in the levels of CACs (Fig. 6.5B). Acute (4d) exposure to 3 ppm CR did not change the levels of Sca-1⁺ cells or CACs (Fig. 6.6A & B). Likewise, female WT mice had no change in the levels of CACs or Sca-1⁺ cells with acute (4d) CR

Table 6.5. Systemic measures in male C57BL/6J mice acutely exposed to either air or crotonaldehyde (CR; 1 or 3 ppm).

| Variable | Exposure Duration (4 days) | | |
|---|----------------------------|--------------|-------------|
| | Air Control | CR (1 ppm) | CR (3 ppm) |
| Change in BWT (g) | -1 ± 0 | -1 ± 0 | -1 ± 0 |
| Heart/BWT (mg/g) | 4.9 ± 0.1 | 5.2 ± 0.1 | 4.9 ± 0.1 |
| Lung/BWT (mg/g) | 5.6 ± 0.1 | 6.0 ± 0.1 | 5.4 ± 0.1 |
| Liver/BWT (mg/g) | 44.6 ± 0.6 | 43.7 ± 1.5 | 43.6 ± 0.9 |
| Kidney/BWT (mg/g) | 12.5 ± 0.1 | 12.7 ± 0.3 | 12.4 ± 0.2 |
| Hematological Measurements | | | |
| White Blood Cell (K/μL) | 1.57 ± 0.13 | 1.96 ± 0.16 | 1.55 ± 0.14 |
| Neutrophils (K/μL) | 0.39 ± 0.06 | 0.62 ± 0.10# | 0.29 ± 0.03 |
| Lymphocytes (K/μL) | 1.13 ± 0.09 | 1.28 ± 0.10 | 1.22 ± 0.11 |
| Monocytes (K/μL) | 0.04 ± 0 | 0.06 ± 0.01 | 0.04 ± 0 |
| Eosinophils (K/μL) | 0.01 ± 0 | 0 ± 0 | 0 ± 0 |
| Basophils (K/μL) | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Red Blood Cell (M/μL) | 8.62 ± 0.10 | 8.71 ± 0.10 | 8.83 ± 0.11 |
| Hemoglobin (g/dL) | 11.7 ± 0.2 | 11.7 ± 0.2 | 11.9 ± 0.1 |
| Hematocrit (%) | 39.2 ± 0.8 | 37.5 ± 0.7 | 42.1 ± 0.5 |
| Mean Corpuscular Volume (fL) | 45.9 ± 0.5 | 43.1 ± 0.5* | 47.7 ± 0.3 |
| Mean Corpuscular Hemoglobin (pg) | 13.6 ± 0.2 | 13.5 ± 0.3 | 13.5 ± 0.1 |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 29.6 ± 0.6 | 31.4 ± 1.1 | 28.3 ± 0.1 |
| Red Cell Distribution Width (%) | 16.9 ± 0.1 | 17.9 ± 0.3* | 17.2 ± 0.3 |
| Platelets (K/μL) | 616 ± 16 | 670 ± 25 | 584 ± 15 |
| Mean Platelet Volume (fL) | 4.3 ± 0 | 4.2 ± 0.1 | 4.3 ± 0 |

Values = mean ± SE (n=9-15 mice per group); Abbr.: **BWT**, body weight; **CR**, crotonaldehyde; *, p<0.05 compared with air control based on Bonferroni's or Dunn's post-test; 0.05≤p≤0.10 compared with air control based on Bonferroni's or Dunn's post-test.

Table 6.6. Plasma biomarkers in male C57BL/6J mice acutely exposed to either air or crotonaldehyde (CR; 1 or 3 ppm).

| Plasma Measurements | Exposure Duration (4 days) | | |
|------------------------------|----------------------------|----------------|----------------|
| | Air Control | CR (1 ppm) | CR (3 ppm) |
| Cholesterol (mg/dL) | 69.97 ± 1.83 | 60.16 ± 1.84* | 74.72 ± 1.89# |
| HDL (mg/dL) | 50.96 ± 1.21 | 44.57 ± 2.31* | 53.10 ± 1.43 |
| LDL (mg/dL) | 4.49 ± 0.37 | 4.32 ± 0.62 | 5.52 ± 0.19 |
| Triglycerides (mg/dL) | 43.76 ± 1.39 | 33.36 ± 2.39* | 41.31 ± 2.22 |
| Albumin (g/dL) | 2.81 ± 0.04 | 2.65 ± 0.05 | 2.85 ± 0.05 |
| Total Protein (g/dL) | 4.76 ± 0.07 | 4.45 ± 0.06* | 5.01 ± 0.08# |
| ALT (U/I) | 23.34 ± 1.25 | 23.74 ± 2.92 | 19.37 ± 0.63 |
| AST (U/I) | 61.99 ± 4.97 | 56.39 ± 6.12 | 52.86 ± 4.05 |
| CK (U/I) | 188.84 ± 21.28 | 127.15 ± 13.94 | 262.70 ± 33.38 |
| LDH (U/I) | 135.90 ± 7.72 | 132.28 ± 11.21 | 141.10 ± 10.36 |
| Creatinine (mg/dL) | 0.28 ± 0.01 | 0.24 ± 0.01# | 0.31 ± 0.01 |

Values = mean ± SE (n=5-10 mice per group); Abbr.: **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **CK**, creatine kinase; **CR**, crotonaldehyde; **HDL**, high-density lipoprotein; **LDH**, lactate dehydrogenase; **LDL**, low-density lipoprotein; *, p<0.05 compared with air control based on Bonferroni's or Dunn's post-test; #, 0.05≤p≤0.10 compared with air control based on Bonferroni's or Dunn's post-test; †, p<0.05 for WT vs TRPA1-null based on Dunn's post-test.

Table 6.7. Systemic measures in female C57BL/6J mice acutely exposed to either air or crotonaldehyde (CR; 1 ppm).

| Variable | Exposure | |
|---|--------------|--------------|
| | Air Control | CR (1 ppm) |
| Change in BWT (g) | -1 ± 0 | -1 ± 0 |
| Heart/BWT (mg/g) | 5.2 ± 0.1 | 5.0 ± 0.1 |
| Lung/BWT (mg/g) | 6.6 ± 0.1 | 6.3 ± 0.2 |
| Liver/BWT (mg/g) | 45.4 ± 1.8 | 44.4 ± 1.4 |
| Kidney/BWT (mg/g) | 13.3 ± 0.4 | 13.1 ± 0.2 |
| Hematological Measurements (n=9-10 mice per group) | | |
| White Blood Cell (K/μL) | 1.30 ± 0.14 | 1.15 ± 0.16 |
| Neutrophils (K/μL) | 0.30 ± 0.05 | 0.26 ± 0.05 |
| Lymphocytes (K/μL) | 0.96 ± 0.10 | 0.86 ± 0.12 |
| Monocytes (K/μL) | 0.03 ± 0 | 0.03 ± 0.01 |
| Eosinophils (K/μL) | 0 ± 0 | 0 ± 0 |
| Basophils (K/μL) | 0 ± 0 | 0 ± 0 |
| Red Blood Cell count (M/μL) | 8.99 ± 0.10 | 9.05 ± 0.13 |
| Hemoglobin (g/dL) | 13.0 ± 0.2 | 13.3 ± 0.1 |
| Hematocrit (%) | 44.4 ± 0.5 | 44.9 ± 0.7 |
| Mean Corpuscular Volume (fL) | 49.4 ± 0.4 | 49.6 ± 0.3 |
| Mean Corpuscular Hemoglobin (pg) | 14.4 ± 0.2 | 14.7 ± 0.3 |
| Mean Corpuscular Hemoglobin Concentration | 29.2 ± 0.5 | 29.7 ± 0.5 |
| Red Cell Distribution Width (%) | 16.5 ± 0.2 | 16.6 ± 0.2 |
| Platelets (K/μL) | 499 ± 16 | 503 ± 10 |
| Mean Platelet Volume (fL) | 4.3 ± 0 | 4.2 ± 0.1 |
| Plasma Measurements (n=7-8 mice per group) | | |
| Cholesterol (mg/dL) | 57.40 ± 4.68 | 54.87 ± 2.66 |
| HDL (mg/dL) | 34.37 ± 1.67 | 35.34 ± 2.55 |
| LDL (mg/dL) | 6.70 ± 0.45 | 6.27 ± 0.40 |

| | | |
|-----------------------------|-------------|-------------|
| Albumin (g/dL) | 2.91 ± 0.03 | 2.92 ± 0.06 |
| Total Protein (g/dL) | 4.54 ± 0.09 | 4.56 ± 0.09 |

Values = mean ± SE. Abbr.: **BWT**, body weight; **CR**, crotonaldehyde; **HDL**, high-density lipoprotein; **LDL**, low-density lipoprotein.

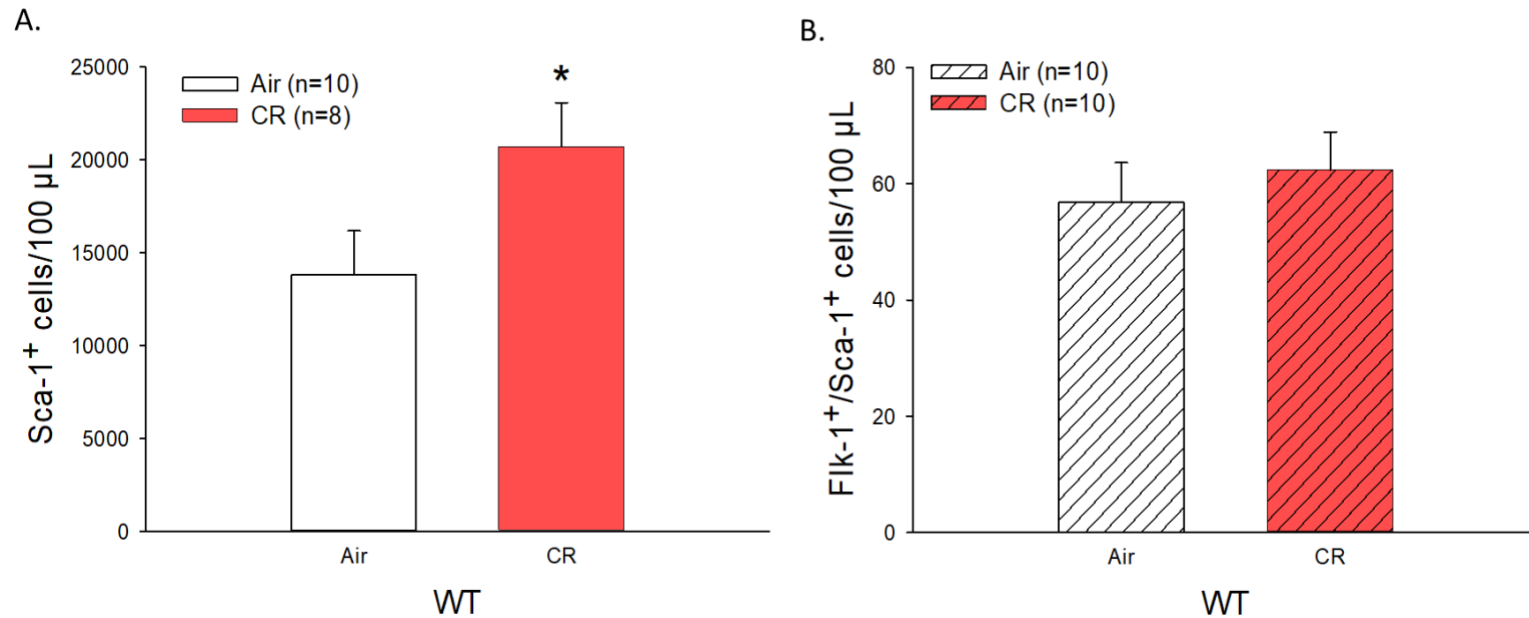


Fig. 6.5. Effects of acute crotonaldehyde (CR; 1 ppm) exposure on circulating stem cells in male mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in male C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or CR (1 ppm, 6h/d) for 4 days. After 4 days of exposure to CR, the level of circulating Sca-1⁺ cells was significantly increased in WT mice (**A**), however, no change in the level of CACs (Flk-1⁺/Sca-1⁺ cells) was detected relative to air-exposed control mice (**B**). Values = mean ± SE (n=10 mice per group); *, p<0.05 compared with air control based on Bonferroni's or Dunn's post-test.

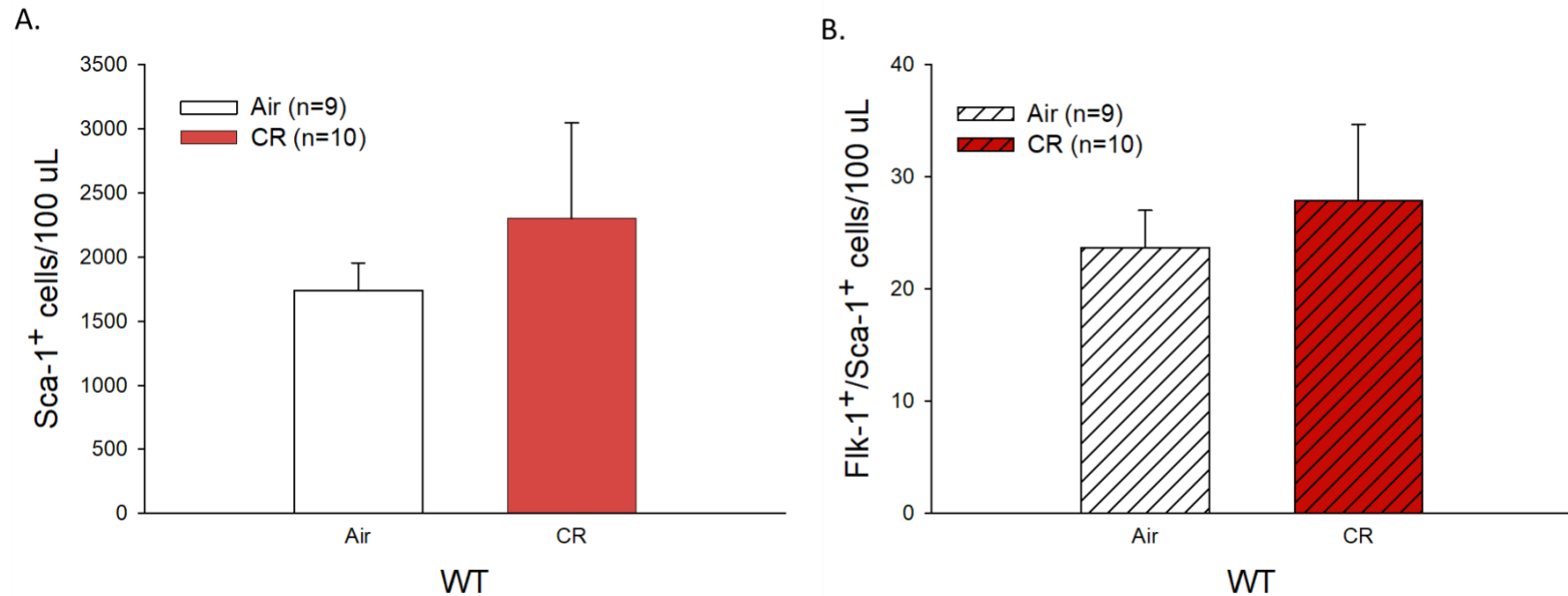


Fig. 6.6. Effects of acute crotonaldehyde (CR; 3 ppm) exposure on circulating stem cells in male mice. Hematopoietic stem cells (Sca-1+) and circulating angiogenic cells (CACs) were measured by flow cytometry in male C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or CR (1 ppm, 6h/d) for 4 days. Levels of circulating Sca-1+ cells (**A**) and CACs (Flk-1+/Sca-1+ cells; **B**) were unchanged in CR-exposed mice relative to air control mice. Values = mean \pm SE (n=9-10 mice per group).

(1 ppm) exposure (Fig. 6.7A & B).

D. Discussion

MCS is a known source of the unsaturated aldehydes acrolein (18-98 $\mu\text{g}/\text{cigarette}$) and CR (10-43 $\mu\text{g}/\text{cigarette}$) (147, 451), and levels of the major urinary metabolites of acrolein (3HPMA) and CR (HPMMA) are significantly increased in smokers versus non-smokers (147, 159). The results of our urinary metabolite studies showed significant increases in both 3HPMA and HPMMA compared with baseline measures. Exposure to MCS and 30%:70% PG:VG produced similar levels of 3HPMA, while 50%:50% PG:VG produced a peak level higher than even that measured after acrolein-alone exposure (151). Conklin et al. (168) showed that levels of acrolein detected in PG-alone aerosol were below the detectable limit, while VG-alone produced levels of acrolein comparable to those measured in flavored e-liquids. Our results, however, indicate that PG is likely driving the production of acrolein, again suggesting that perhaps PG and VG in combination are capable of producing levels of aldehydes that vary compared with PG or VG alone. Regardless, it seems clear that the proportion of PG:VG used in an e-cig device can create significant variation in the aldehydes produced. Conversely, while levels of HPMMA were not below the limit of detection in 50%:50% PG:VG-exposed mice, they were very low and not significantly different from baseline. Previous studies have reported little if any generation of CR in e-cigs (96, 168), so HPMMA should not be used as a biomarker of e-cig use. HPMMA is, however, increased after exposure to MCS, meaning HPMMA would be a more selective biomarker for MCS exposure. Chronic exposure to CR-alone showed a rapid increase in urinary HPMMA, with the level after 11 weeks of exposure exceeding that of MCS more than 5-fold within the first three hours post-exposure. This supports

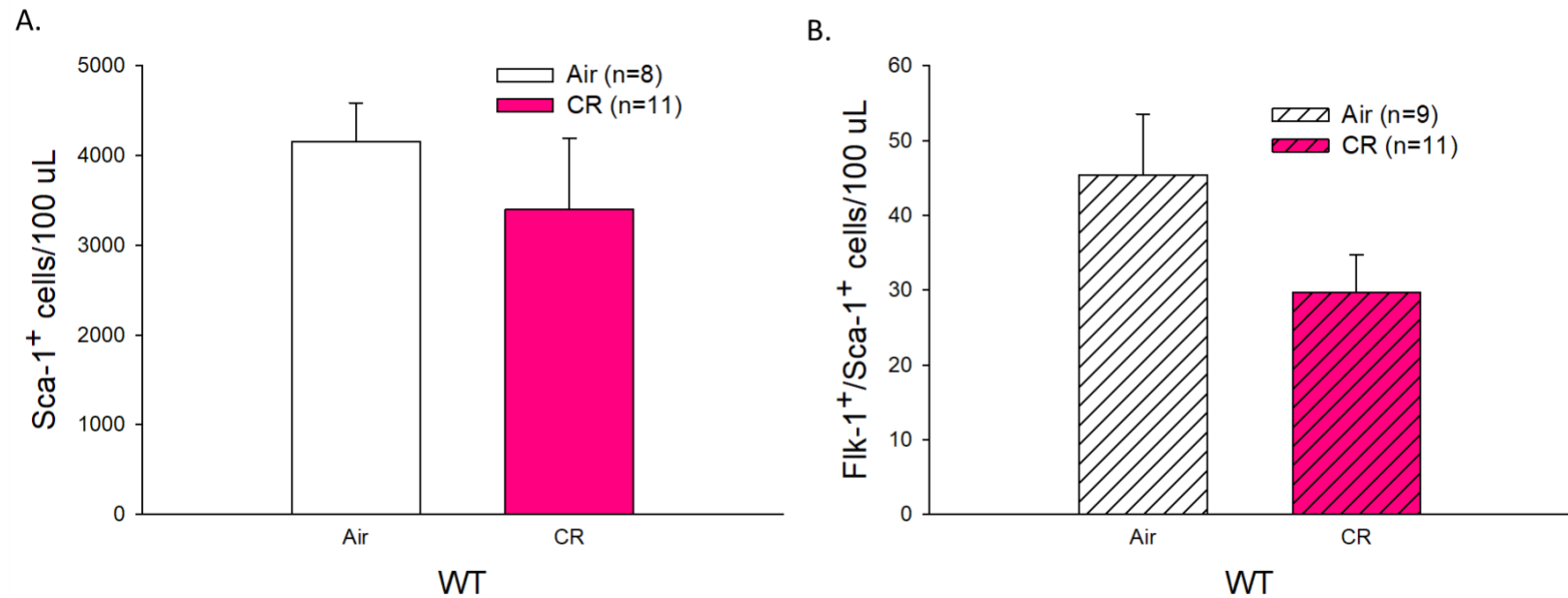


Fig. 6.7. Effects of acute crotonaldehyde (CR; 1 ppm) exposure on circulating stem cells in female mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CAC) were measured by flow cytometry gating in female C57BL/6J (wild type, WT) mice exposed to HEPA-filtered air or CR (1 ppm, 6h/d) for 4 days. Levels of circulating Sca-1⁺ cells (A) and CACs (Flk-1⁺/Sca-1⁺ cells; B) were unchanged in CR-exposed mice relative to air control mice. Values = mean \pm SE (n=8-11 mice per group).

previous findings that levels of CR in MCS are not as high as levels of acrolein but that there is a significant level of CR in cigarettes that may contribute to the cardiovascular effects of smoking.

A number of epidemiological and experimental studies have linked acrolein exposure with increased CVD risk. Previous studies in our group have shown that acrolein contributes to the development of CVD through CAC suppression (74, 151), platelet activation (450), and endothelium dysfunction (150). However, acute (4d; 1 ppm) exposure to acrolein produced no systemic toxicity as measured by CBC or measured in plasma biomarkers (74), and even chronic exposure (12 weeks; 1 ppm) only showed significant changes in leukocytes (151). The current acute (4d) study shows significant decreases in leukocytes, but only minimal changes in plasma biomarkers, and while we did not see a significant decrease in levels of CACs as was previously seen after acrolein exposure (74, 151), there was a trend towards decreased levels that likely did not reach significance due to our small sample size (n=4 air; n=3 acrolein). Although the change in leukocytes seen in the current acute study (1 ppm) vary from the results of the previous study (74), they support the findings of the chronic study (151), indicating that the variation between the acute studies may simply be due to the use of a different set of mice. However, the significant decrease (74) and trending decrease in CACs in the acute studies likely indicate that acrolein primarily induces injury in the endothelium before inducing more systemic changes, although these changes may still occur at a time point earlier than 12 weeks.

Despite the physical and chemical similarities and the shared exposure sources between acrolein and CR in addition to the well-recognized toxicity of CR, the chronic

toxicity of inhaled CR is not well studied. To our knowledge, these studies are the first to evaluate the systemic and vascular effects of acute and chronic CR inhalation exposure. Although CR is known to trigger inflammation (91), the current data do not show changes in circulating leukocytes or specific immune cell differentials in either acute (1 or 3 ppm) or chronic exposures, an outcome differing from that after acute (4d, 1 ppm) and chronic acrolein exposure (12 weeks; 1 ppm) (74, 151). Of course, CR exposure could lead to the suppression of immune function (452, 453) independent of leukocyte numbers as described for alveolar macrophages following acrolein exposure (454). However, these other studies of CR or acrolein are hard to compare with the current studies due to the high-level CR doses given by intratracheal installation that led to mortality (452) or to the use of *in vitro* methods (453, 454). From the inhalation studies presented here, it can be inferred that the immune system overall appears a less sensitive target of CR exposure than of acrolein.

In other systemic measures, chronic CR exposure results in dyslipidemic changes through increases in both cholesterol and triglycerides. High levels of cholesterol and triglycerides are known risk factors of ischemic (455) and coronary heart disease (456). Triglycerides release pre-formed mediators of oxidative stress that influence endothelial cell function by stimulating intracellular production of reactive oxygen species (457), inducing endothelium dysfunction (458, 459), and promoting atherosclerotic plaques (460). Although levels of both cholesterol and triglycerides increased in this chronic study, neither biomarker increased after acute (4d; 1 ppm) exposure. In fact, both cholesterol and triglycerides significantly decrease in the CR-exposed group compared with air-exposed control mice, indicating that chronic increases in lipids are not the primary change initiated by CR exposure.

Over the past decade, studies in our group have shown that exposures of mice and humans to air pollutants (particulate matter (PM_{2.5}), acrolein) lead to the suppression of CACs (71, 73, 74), yet no changes in CACs were observed in these studies. It can therefore be inferred that 1 ppm CR is not as potent of a stimulus of CAC suppression as is either PM_{2.5} or acrolein (1 ppm).

These studies also examined the effect of sex on the measured CR-induced outcomes. Female mice acutely exposed to CR (4d; 1 ppm) showed no significant changes in any systemic or vascular biomarkers, indicating that the effects of CR exposure are likely sex-dependent. Epidemiological studies seem to support this outcome; analysis of male versus female smokers in the National Health and Nutrition Examination Study (NHANES) 2005-2006 and 2011-2012 cohorts showed significant differences in urinary HPMMA (159). As previous studies in acrolein-exposed mice also show that the effects of acrolein are sex-dependent (269), it may be that there are sex differences in the pharmacodynamics and pharmacokinetics in the metabolism of the unsaturated aldehydes. Additional studies of acrolein and CR exposure in both male and female mice should work to identify the mechanism of this female-dependent protection with the idea that this clarification could be used in the development of effective interventions and treatments for unsaturated aldehyde exposure.

In summary, the studies presented in this chapter overall confirm the results of previous work examining the effects of acrolein exposure and have demonstrated the systemic and hematological effects of both acute and chronic CR exposure. In particular, chronic CR exposure was shown to increase levels of plasma biomarkers indicative of dyslipidemia and possibly subsequent promotion of atherosclerotic development.

Comparisons of the outcomes between acrolein and CR seem to demonstrate that acrolein is more toxic than CR, at least at the concentrations and durations tested. However, the presence of high levels of CR in MCS and other sources, as well as the lack of previous research, demonstrate the need for a better understanding of how CR exposure contributes to the cardiovascular pathology of real-world combustion-related exposures. Furthermore, the sex-dependence shown in relation to acute CR exposure supports the idea of sex-dependence in the cardiovascular effects of unsaturated aldehyde exposures, an area that requires additional study. As exposures to acrolein and CR remain a daily occurrence for most of the global population, these studies confirm previous research and provide novel insight into the role of unsaturated aldehydes in the development of CVD, specifically implicating chronic CR exposure as a risk factor for CVD.

CHAPTER VII

ROLES OF EXTRACELLULAR SUPEROXIDE DISMUTASE AND TRANSIENT RECEPTOR POTENTIAL ANKYRIN 1 IN THE CARDIOVASCULAR EFFECTS OF TOBACCO PRODUCTS AND CONSTITUENT ALDEHYDES

A. Introduction

One of the mechanisms proposed by Brook et al. (16) to explain how air pollutants may contribute to the development of cardiovascular disease (CVD) is the development of systemic oxidative stress and inflammation within the vasculature. In this hypothesis, oxidative stress results from overproduction of reactive oxygen species (ROS), which are highly reactive, oxygen-containing molecules (16, 40-43). ROS have been shown to play a beneficial role when maintained at normal levels (42-44), but excess production can lead to the development of oxidative stress (40-42, 44, 47), which has been linked to incidence of CVD (42, 48-52). Mechanistically, the ROS-related development of CVD is at least in part mediated by changes in the bioavailability of nitric oxide (NO) and the development of endothelium dysfunction (40-42). The enzyme superoxide dismutase (SOD) indirectly plays an important role in regulating levels of NO in the blood; SOD catalyzes the dismutation of superoxide, but when the enzyme is inactivated, the free superoxide molecules can bind with NO and cause a decrease in free NO levels (40, 41, 49, 50, 53).

The subsequent imbalance of vasoactive factors in the vasculature can result in endothelium dysfunction (40, 41), which is one of the earliest vascular changes in a number of CVDs (54-58). Although ROS have been identified as therapeutic targets for the treatment of CVD (59-61), more research is needed to better understand the relationship between these vascular changes and disease development.

One of the many channels activated by ROS is the transient receptor potential ankyrin 1 (TRPA1) channel (263-266, 461, 462). The TRPA1 channel has also been shown to be activated by a number of other stimuli, including physical factors (260, 262) and air pollutants including diesel exhaust, mainstream cigarette smoke (MCS), and aldehydes (127, 260, 262, 266, 268-270). TRPA1 is primarily recognized as a pain (262, 272, 279) or irritant (262, 264, 279, 286) receptor, but a number of other studies have demonstrated the role of TRPA1 in vasodilation (127, 260, 265, 266, 270, 272). Additionally, Conklin et al. (269) demonstrated that the TRPA1 channel plays an important role in the cardiopulmonary toxicity and lethality induced by high-level exposure to the aldehyde acrolein, and several other studies have also shown that the negative cardiovascular effects of inhaled acrolein are TRPA1-dependent (261, 289, 290). However, the role of TRPA1 in the cardiovascular toxicity of other aldehydes, including crotonaldehyde (CR), is not well explored. Thus, the goal of these studies was to examine the roles of SOD, specifically extracellular SOD (ecSOD), and the TRPA1 channel in the mechanism through which constituents of personal air pollutant exposures mediate cardiovascular toxicity.

B. Experimental Procedures

1. Exposures

Animals were exposed to compounds or constituents via full-body inhalation as

described in Chapter II, Sections B.2. Upon completion of the exposure, mice were euthanized and tissues collected as described in Chapter II, Section B.5.

2. Urine Collection

Urine samples were collected post-exposure as described in Chapter II, Section B.3.

3. Urine Metabolite Quantification

Collected urine was used to quantify levels of volatile organic compound (VOC) and tobacco alkaloid metabolites using analytical methods as described in Chapter II, Section B.4.

4. Complete Blood Cell Count (CBC)

A sample of whole blood was collected from each mouse after the final day of exposure for a complete blood cell count (CBC) as described in Chapter II, Section B.6.

3. Plasma Biomarkers Quantification

Plasma was prepared from each sample for an analysis of a number of biomarkers as described in Chapter II, Section B.7.

4. Circulating Angiogenic Cell (CAC) Quantification

Flow cytometry was used to analyze the number of circulating angiogenic cells (CACs) in each sample as described in Chapter II, Section B.8.

5. Statistical Analyses

Statistical analyses were performed as described in Chapter II, Section B.10.

C. Results

1. Acute Exposure to MCS and the Role of TRPA1

Because TRPA1 activation and neurogenic inflammation are induced by MCS, we performed acute exposures to MCS (4 days; 50% of smoke of 12 KY Reference 3R4F cigarettes) using TRPA1-null male mice. Similar to the effects seen in MCS-exposed WT mice, TRPA-1 null mice showed a decreased final body weight (-11%; $p < 0.001$), but no changes in organ/body weight ratios (Table 7.1). Also similar to the WT mice, overall leukocyte count was significantly decreased with MCS exposure (-51%; $p < 0.001$), although this decrease was driven by decreases in both lymphocytes and monocytes (-34%; $p = 0.004$ and -40%; $p = 0.02$, respectively) (Table 7.2). MCS exposure otherwise had limited effects on CBC measures, inducing only a slight but significant increase in red cell distribution width (+2%; $p = 0.03$) and a modest decrease in platelet count (-16%; $p = 0.06$) (Table 7.2). Measures of liver (ALT, AST), muscle (CK), and LDH enzymes were unaffected by exposure, but exposed TRPA1-null mice showed a modest decrease in LDL (-23%; $p = 0.05$) and a modest increase in albumin (+9%; $p = 0.07$) (Table 7.1).

2. Acute Exposure to Crotonaldehyde and the Role of TRPA1

Because TRPA1 activation and neurogenic inflammation are induced by MCS and acrolein (261), we performed acute exposures to CR (4 days; 1 ppm) using TRPA1-null male mice. In contrast to the effects observed in CR-exposed male WT mice, TRPA1-null mice had no systemic changes in CBC (Table 7.3) yet did have a decreased kidney/body weight ratio (-9%; $p < 0.001$) relative to air-exposed control mice (Table 7.3). Plasma levels of ALT, AST, CK, and LDH enzymes, albumin, and creatinine were unchanged between the air- and CR-exposed TRPA1-null mice (Table 7.4). Cholesterol was decreased by CR exposure in the TRPA1-null mice (-17%; $p = 0.003$; a change also observed in WT mice), a change due to decreased levels of LDL (-49%; $p < 0.001$) rather than in HDL level as

Table 7.1. Systemic parameters of male TRPA1-null mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 12 KY Reference 3R4F cigarettes).

| Variable | Exposure (4 days) | |
|------------------------------|-------------------|-----------------|
| | Air Control | MCS |
| Final BWT (g) | 33 ± 0† | 29 ± 1*† |
| Heart/BWT (mg/g) | 5.5 ± 0.1 | 5.6 ± 0.1† |
| Lung/BWT (mg/g) | 6.1 ± 0.2 | 6.0 ± 0.1 |
| Liver/BWT (mg/g) | 46.6 ± 2.2 | 47.7 ± 2.2† |
| Kidney/BWT (mg/g) | 14.2 ± 0.6 | 13.4 ± 0.4 |
| Plasma Measurements | | |
| Cholesterol (mg/dL) | 81.83 ± 4.48 | 78.00 ± 3.57 |
| HDL (mg/dL) | 45.83 ± 2.26 | 48.29 ± 3.41 |
| LDL (mg/dL) | 26.47 ± 2.21 | 20.34 ± 0.78# |
| Triglycerides (mg/dL) | 47.67 ± 1.82† | 46.86 ± 1.65† |
| Albumin (g/dL) | 2.57 ± 0.03† | 2.79 ± 0.06#† |
| Total Protein (g/dL) | 5.07 ± 0.11 | 5.01 ± 0.07 |
| ALT (U/I) | 12.67 ± 2.16† | 12.00 ± 1.48† |
| AST (U/I) | 44.17 ± 3.49 | 48.43 ± 10.95 |
| CK (U/I) | 112.67 ± 11.78† | 144.73 ± 52.39 |
| LDH (U/I) | 142.67 ± 9.64† | 134.14 ± 10.29† |

Values = mean ± SE (n=6-7 mice per group); HDL & LDL compared using Rank Sum Test. Abbr.: **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **BWT**, body weight; **CK**, creatine kinase; **HDL**, high-density lipoprotein; **LDH**, lactate dehydrogenase; **LDL**, low-density lipoprotein; **MCS**, mainstream cigarette smoke; **TRPA1**, transient receptor potential ankyrin 1;

*, $p < 0.05$ compared with air control based on Bonferroni's or Dunn's post-test; #, $0.05 \leq p \leq 0.10$ compared with air control based on Bonferroni's or Dunn's post-test; †, $p < 0.05$ for WT vs TRPA1-null based on Dunn's post-test.

Table 7.2. Hematological measures of male TRPA1-null mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 12 KY Reference 3R4F cigarettes).

| Hematological Measurements | Exposure (4 days) | |
|--|-------------------|---------------|
| | Air Control | MCS |
| White Blood Cell (K/uL) | 3.87 ± 0.41† | 1.91 ± 0.17*† |
| Neutrophils (K/uL) | 1.65 ± 0.34 | 0.46 ± 0.04 |
| Lymphocytes (K/uL) | 2.13 ± 0.20† | 1.40 ± 0.15*† |
| Monocytes (K/uL) | 0.09 ± 0.01† | 0.05 ± 0.01*¶ |
| Eosinophils (K/uL) | 0 ± 0 | 0 ± 0 |
| Basophils (K/uL) | 0 ± 0 | 0 ± 0 |
| Red Blood Cell (M/uL) | 8.91 ± 0.24† | 8.88 ± 0.19 |
| Hemoglobin (g/dL) | 13.1 ± 0.3† | 13.3 ± 0.2† |
| Hematocrit (%) | 44.0 ± 1.0† | 44.9 ± 1.0† |
| Mean Corpuscular Volume (fL) | 49.5 ± 0.8† | 50.6 ± 0.3† |
| Mean Corpuscular Hemoglobin (pg) | 14.7 ± 0.3† | 15.0 ± 0.1† |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 29.7 ± 0.4 | 29.7 ± 0.2† |
| Red Cell Distribution Width (%) | 18.5 ± 0.6¶ | 17.4 ± 0.3* |
| Platelets (K/uL) | 880 ± 108 | 648 ± 36#† |
| Mean Platelet Volume (fL) | 4.5 ± 0 | 4.3 ± 0.1 |

Values = mean ± SE (n=6-7 mice per group); Abbr.: **MCS**, mainstream cigarette smoke; **TRPA1**, transient receptor potential ankyrin 1; *, p<0.05 compared with air control based on Bonferroni's or Dunn's post-test; #, 0.05≤p≤0.10 compared with air control based on Bonferroni's or Dunn's post-test; †, p<0.05 for WT vs TRPA1-null based on Dunn's post-test; ¶, 0.05≤p≤0.10 for WT vs TRPA1-null based on Dunn's post-test.

Table 7.3. Systemic measures in male TRPA1-null mice acutely exposed to either air or crotonaldehyde (CR; 1 ppm).

| Variable | Exposure Duration (4 days) | |
|---|----------------------------|-------------|
| | Air Control | CR (1 ppm) |
| Change in BWT (g) | -1 ± 1 | -1 ± 0 |
| Heart/BWT (mg/g) | 5.3 ± 0.2 | 5.0 ± 0.2 |
| Lung/BWT (mg/g) | 5.7 ± 0.2 | 5.4 ± 0.2 |
| Liver/BWT (mg/g) | 47.8 ± 2.0† | 47.8 ± 1.0† |
| Kidney/BWT (mg/g) | 14.2 ± 0.4† | 12.9 ± 0.2* |
| Hematological Measurements | | |
| White Blood Cell (K/μL) | 1.70 ± 0.24 | 1.57 ± 0.27 |
| Neutrophils (K/μL) | 0.59 ± 0.12 | 0.47 ± 0.09 |
| Lymphocytes (K/μL) | 1.06 ± 0.12 | 1.06 ± 0.18 |
| Monocytes (K/μL) | 0.05 ± 0.01 | 0.08 ± 0.04 |
| Eosinophils (K/μL) | 0 ± 0 | 0 ± 0 |
| Basophils (K/μL) | 0 ± 0 | 0 ± 0 |
| Red Blood Cell (M/μL) | 8.40 ± 0.13 | 8.72 ± 0.10 |
| Hemoglobin (g/dL) | 11.6 ± 0.2 | 12.1 ± 0.1 |
| Hematocrit (%) | 37.8 ± 0.7 | 39.5 ± 0.4 |
| Mean Corpuscular Volume (fL) | 45.0 ± 0.3 | 45.3 ± 0.3† |
| Mean Corpuscular Hemoglobin (pg) | 13.9 ± 0.1 | 13.8 ± 0.1 |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 30.8 ± 0.2 | 30.6 ± 0.3 |
| Red Cell Distribution Width (%) | 18.3 ± 0.4† | 17.7 ± 0.3 |
| Platelets (K/μL) | 869 ± 51† | 862 ± 56† |
| Mean Platelet Volume (fL) | 4.5 ± 0.1† | 4.6 ± 0.1† |

Values = mean ± SE (n=9-15 mice per group); Abbr.: **BWT**, body weight; **CR**, crotonaldehyde; **TRPA1**, transient receptor potential ankyrin 1; *, p<0.05 compared with air control based on Bonferroni's or Dunn's post-test; †, p<0.05 for WT vs TRPA1-null based on

Dunn's post-test.

Table 7.4. Plasma biomarkers in male TRPA1-null mice acutely exposed to either air or crotonaldehyde (CR; 1 ppm).

| Plasma Measurements | Exposures (4 days) | |
|------------------------------|--------------------|----------------|
| | Air Control | CR (1 ppm) |
| Cholesterol (mg/dL) | 81.39 ± 3.13† | 67.27 ± 3.48* |
| HDL (mg/dL) | 49.95 ± 2.89 | 47.34 ± 2.80 |
| LDL (mg/dL) | 8.73 ± 0.96† | 4.49 ± 0.52* |
| Triglycerides (mg/dL) | 40.77 ± 3.60 | 27.39 ± 1.66 |
| Albumin (g/dL) | 2.75 ± 0.10 | 2.77 ± 0.07 |
| Total Protein (g/dL) | 5.11 ± 0.17† | 4.83 ± 0.11† |
| ALT (U/I) | 28.91 ± 1.43 | 33.71 ± 5.31 |
| AST (U/I) | 70.62 ± 4.13 | 69.88 ± 8.82 |
| CK (U/I) | 222.70 ± 27.59 | 233.81 ± 31.69 |
| LDH (U/I) | 138.03 ± 7.35 | 160.28 ± 12.70 |
| Creatinine (mg/dL) | 0.25 ± 0.01 | 0.24 ± 0.01 |

Values = mean ± SE (n=5-6 mice per group); Abbr.: **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **CK**, creatine kinase; **CR**, crotonaldehyde; **HDL**, high-density lipoprotein; **LDH**, lactate dehydrogenase; **LDL**, low-density lipoprotein; **TRPA1**, transient receptor potential ankyrin 1; *, p<0.05 compared with air control based on Bonferroni's or Dunn's post-test; #, 0.05≤p≤0.10 compared with air control based on Bonferroni's or Dunn's post-test; †, p<0.05 for WT vs TRPA1-null based on Dunn's post-test.

observed in WT mice (Table 7.4). As with the WT mice, TRPA1-null mice acutely exposed to 1 ppm CR had no change in the levels of circulating Sca-1⁺ cells or Flk-1⁺/Sca-1⁺ cells (Fig. 7.1A & B).

3. Urinary Metabolites in MCS-Exposed WT and ecSOD-Tg Mice

The antioxidant enzyme superoxide dismutase (SOD) catalyzes the dismutation of superoxide molecules to prevent their binding with nitric oxide and the subsequent formation of peroxynitrite (40-42, 53). Peroxynitrite can disrupt the proper production of nitric oxide, which contributes to the development of endothelium dysfunction (40, 41). This dysfunction is one of the early vascular changes noted in a number of CVDs (54-57), but increased levels of SOD, particularly extracellular SOD (ecSOD), have been shown to be protective in cardiac tissues (463). To better understand the role of ecSOD in the cardiovascular-related effects of MCS, we measured urinary metabolites by UPLC-MS/MS and GC-MS/MS in mice exposed to a known number of cigarettes. For this, male mice overexpressing ecSOD (ecSOD-Tg; Tg) and their wild type littermates (WT) were exposed to MCS (50% of smoke of 12 or 6 KY Reference 3R4F cigarettes, 6h/day, 4d), and urine was immediately collected after the exposure sessions. Urinary levels of 3-hydroxypropylmercapturic acid (3HPMA) were not significantly different between WT and Tg mice at baseline (Fig. 7.2A). Likewise, levels of 3HPMA after the first three hours post-MCS exposure were not significantly different from baseline, nor were levels different between background (WT or Tg) or between number of cigarettes (12 or 6) (Fig. 7.2A). Conversely, urinary HPMMA levels were significantly increased in both WT (p=0.02) and Tg (p=0.003) mice exposed to 12 cigarettes and WT (p=0.02) and Tg (p=0.006) mice exposed to 6 cigarettes compared with baseline (Fig 7.2B). However, there were again no

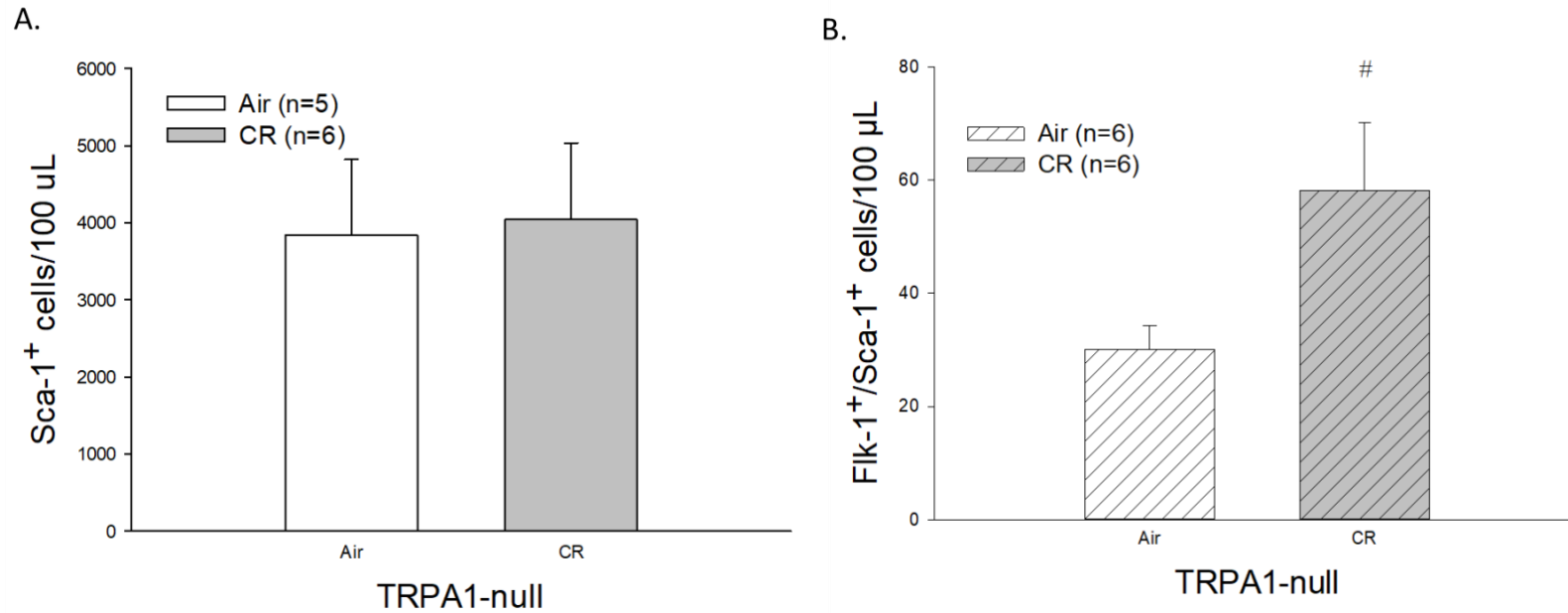


Fig. 7.1. Effects of acute crotonaldehyde (CR; 1 ppm) exposure on circulating stem cells in male TRPA1-null mice.

Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in male TRPA1-null mice exposed to HEPA-filtered air or CR (1 ppm, 6h/d) for 4 days. After 4 days of exposure to CR, TRPA1-null mice had no change in the level of Sca-1⁺ cells (**A**) but had a modest increase in the level of CACs (Flk-1⁺/Sca-1⁺ cells (**B**) compared with air-exposed control. Values = mean ± SE (n=5-6 mice per group); #, 0.05 ≤ p ≤ 0.10 vs air control.

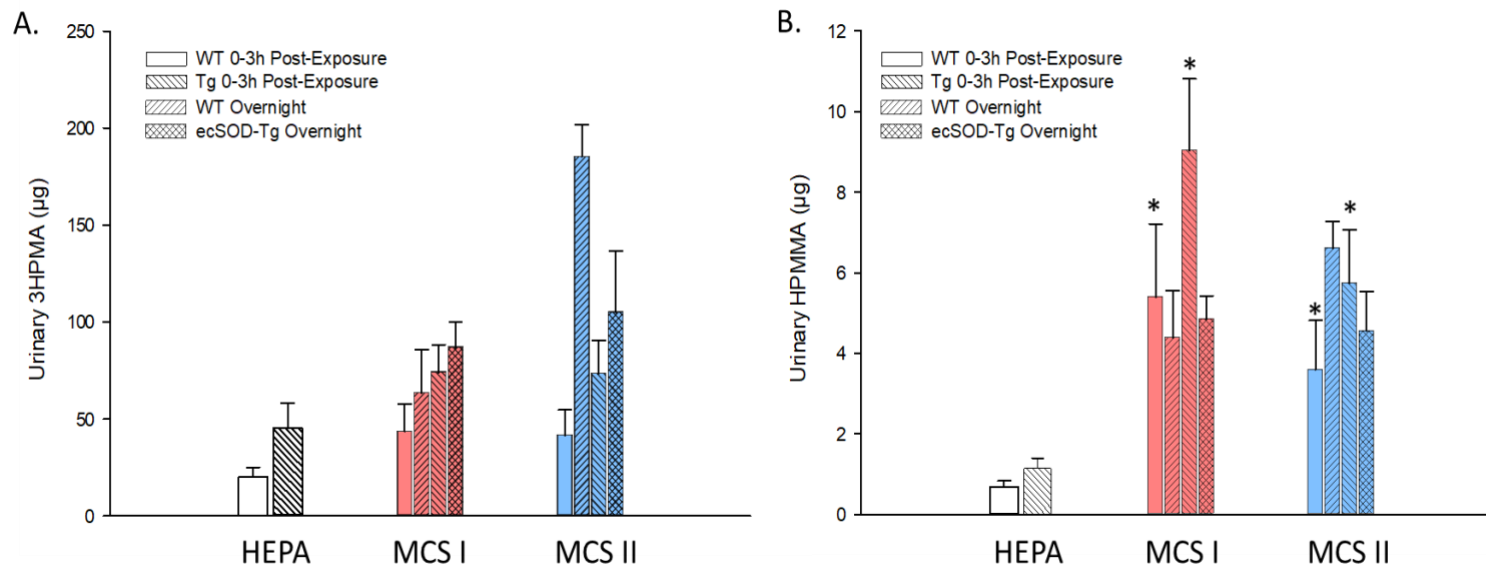


Fig. 7.2. Urinary 3HPMA & HPMMA levels after acute mainstream cigarette smoke (MCS) exposure. Male WT and ecSOD-Tg mice were exposed (6h) to mainstream cigarette smoke (MCS; MCS I: 50% of smoke of 12 KY Reference 3R4F cigarettes; MCS II: 50% of smoke of 6 KY Reference 3R4F cigarettes) after which urine was collected at 0–1, 1–2, 2–3, and 3–16h (overnight) and 3-hydroxypropylmercapturic acid (3HPMA; **A**) and 3-hydroxy-1-methylpropylmercapturic acid (HPMMA; **B**) quantified. Values are mean \pm SE (n=3-5 mice per group). **Abbr.:** ecSOD-Tg; extracellular superoxide dismutase transgenic; **HEPA**, high-efficiency

particulate air; **3HPMA**, 3-hydroxypropylmercapturic acid; **HPMMA**, 3-hydroxy-1-methylpropylmercapturic acid; **MCS**, mainstream cigarette smoke; **WT**, wild type. *, $p < 0.05$ compared with same strain HEPA control.

significant differences in HPMMA between WT and Tg mice exposed to the same number of cigarettes (Fig. 7.2B). Levels of total urinary nicotine metabolites were below the limit of detection in HEPA-exposed mice, and mice exposed to either 6 or 12 cigarettes showed significant increases in the first three hours post-exposure regardless of background (WT: $p=0.02$ for both; Tg: $p<0.001$ for both) (Fig. 7.3). Levels were not different, however, between mice of the same background exposed to the same number of cigarettes (Fig. 7.3).

4. Acute Exposure to MCS and the Role of ecSOD

Because of the protective cardiopulmonary effects seen with overexpression of the antioxidant enzyme ecSOD (463-465), we tested for the potential benefit of this overexpression in biomarkers of systemic and hematological injury in mice exposed to MCS (50% of smoke of 6 or 12 3R4F KY reference cigarettes). Tg mice acutely (4d) exposed to 6 cigarettes showed a greater final body weight compared with their 6 cigarette-exposed WT littermates (+6%; $p=0.07$) (Table 7.5), while no change was seen between WT and Tg mice exposed to 12 cigarettes (Table 7.6). The lung/body weight ratio, however, was significantly lower in Tg mice exposed to 12 cigarettes compared with 12 cigarette-exposed WT mice (-20%; $p=0.02$) (Table 7.6); only the liver/body weight ratio was different between WT and Tg mice exposed to 6 cigarettes, with Tg mice showing a modestly lower ratio (-7%; $p=0.07$) (Table 7.5). Acute exposure showed minimal differences in both CBC and plasma biomarkers between WT and Tg mice exposed to the same number of cigarettes but considerable differences in mice of the same background exposed to a different number of cigarettes. For example, in mice exposed to 6 cigarettes, lymphocyte counts were lower in Tg mice (-23%; $p=0.08$) (Table 7.5), but no differences were noted in mice exposed to 12 cigarettes (Table 7.6). However, both WT and Tg mice

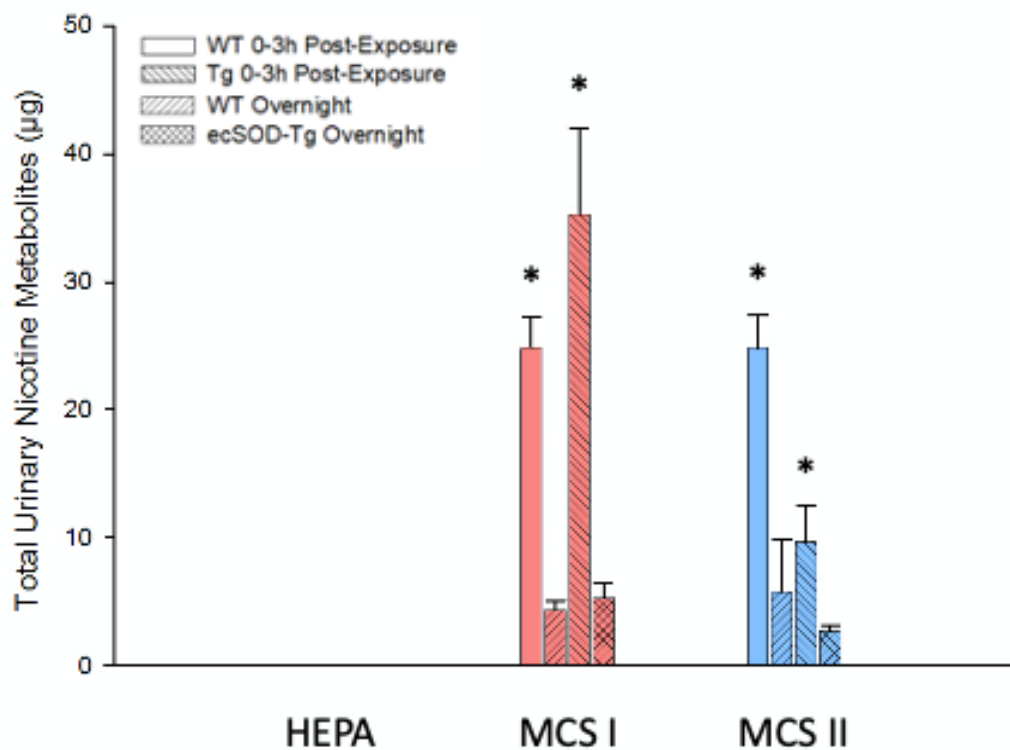


Fig. 7.3. Total urinary nicotine metabolite levels after acute mainstream cigarette smoke (MCS) exposure. Male WT and ecSOD-Tg mice were exposed (6h) to mainstream cigarette smoke (MCS; MCS I: 50% of 12 KY Reference 3R4F cigarettes; MCS II: 50% of smoke of 6 KY Reference 3R4F cigarettes) after which urine was collected at 0–1, 1–2, 2–3, and 3–16h (overnight) and total urinary nicotine metabolites (nicotine, cotinine, and trans-3'-hydroxycotinine) quantified. Values are mean \pm SE (n=3-5 mice per group). **Abbr.:** ecSOD-Tg; extracellular superoxide dismutase transgenic; **HEPA**, high-efficiency particulate air; **MCS**, mainstream cigarette smoke; **WT**, wild type. *, p<0.05 compared with same strain HEPA control.

Table 7.5. Systemic changes in male WT or ecSOD-Tg mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 6 KY Reference 3R4F cigarettes).

| Variable | MCS (6 cigs, 4d) | |
|---|------------------|---------------|
| | WT | ecSOD-Tg |
| Final BWT (g) | 30 ± 1 | 32 ± 1# |
| Heart/BWT (mg/g) | 5.4 ± 0.3 | 4.7 ± 0.1 |
| Lung/BWT (mg/g) | 5.3 ± 0.3 | 5.1 ± 0.1 |
| Liver/BWT (mg/g) | 42.7 ± 1.1 | 39.7 ± 0.6# |
| Hematological Measurements | | |
| White Blood Cell (K/μL) | 3.83 ± 0.63† | 3.55 ± 0.51¶ |
| Neutrophils (K/μL) | 1.27 ± 0.53 | 1.55 ± 0.49¶ |
| Lymphocytes (K/μL) | 2.48 ± 0.34† | 1.91 ± 0.19#¶ |
| Monocytes (K/μL) | 0.07 ± 0.01 | 0.08 ± 0.02¶ |
| Eosinophils (K/μL) | 0.01 ± 0 | 0.01 ± 0.01 |
| Basophils (K/μL) | 0 ± 0 | 0 ± 0 |
| Red Blood Cell (M/μL) | 8.35 ± 0.35 | 8.54 ± 0.16¶ |
| Hemoglobin (g/dL) | 12.3 ± 0.9 | 12.6 ± 0.5¶ |
| Hematocrit (%) | 36.3 ± 1.4 | 37.3 ± 0.8¶ |
| Mean Corpuscular Volume (fL) | 43.5 ± 0.2 | 43.7 ± 0.2 |
| Mean Corpuscular Hemoglobin (pg) | 14.7 ± 0.6 | 14.8 ± 0.4 |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 33.8 ± 1.5 | 33.9 ± 0.7 |
| Red Cell Distribution Width (%) | 18.2 ± 0.2 | 18.0 ± 0.2 |
| Platelets (K/μL) | 776 ± 57 | 788 ± 24 |
| Mean Platelet Volume (fL) | 4.7 ± 0 | 4.7 ± 0 |

Values = mean ± SE (n=5 mice per group); Abbr.: **BWT**, body weight; **ecSOD**, extracellular superoxide dismutase; **ecSOD-Tg**,

extracellular superoxide dismutase transgenic; **MCS**, mainstream cigarette smoke; **WT**, wild type; *, $p < 0.05$ compared with WT based on Bonferroni's or Dunn's post-test; #, $0.05 \leq p \leq 0.10$ compared with WT based on Bonferroni's or Dunn's post-test; †, $0.05 \leq p \leq 0.10$ for 12 vs 6 cigarettes in WT mice based on Dunn's post-test; ¶, $0.05 \leq p \leq 0.10$ for 12 vs 6 cigarettes in ecSOD-Tg mice based on Dunn's post-test.

Table 7.6. Systemic changes in male WT or ecSOD-Tg mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 12 KY Reference 3R4F cigarettes).

| Variable | MCS (12 cigs, 4d) | |
|---|-------------------|-------------|
| | WT | ecSOD-Tg |
| Final BWT (g) | 31 ± 0 | 32 ± 1 |
| Heart/BWT (mg/g) | 4.8 ± 0.2 | 4.1 ± 0.5 |
| Lung/BWT (mg/g) | 6.1 ± 0.3 | 4.8 ± 0.5* |
| Liver/BWT (mg/g) | 39.9 ± 1.4 | 40.9 ± 1.1 |
| Hematological Measurements | | |
| White Blood Cell (K/μL) | 1.38 ± 0.24 | 1.25 ± 0.21 |
| Neutrophils (K/μL) | 0.43 ± 0.12 | 0.40 ± 0.05 |
| Lymphocytes (K/μL) | 0.90 ± 0.11 | 0.81 ± 0.19 |
| Monocytes (K/μL) | 0.04 ± 0.01 | 0.04 ± 0.01 |
| Eosinophils (K/μL) | 0 ± 0 | 0 ± 0 |
| Basophils (K/μL) | 0 ± 0 | 0 ± 0 |
| Red Blood Cell (M/μL) | 7.66 ± 0.39 | 7.26 ± 0.37 |
| Hemoglobin (g/dL) | 10.8 ± 0.3 | 10.3 ± 0.7 |
| Hematocrit (%) | 33.5 ± 1.7 | 31.6 ± 1.8 |
| Mean Corpuscular Volume (fL) | 43.7 ± 0.1 | 43.5 ± 0.3 |
| Mean Corpuscular Hemoglobin (pg) | 14.1 ± 0.4 | 14.1 ± 0.3 |
| Mean Corpuscular Hemoglobin Concentration (g/dL) | 32.3 ± 0.9 | 32.2 ± 0.7 |
| Red Cell Distribution Width (%) | 18.6 ± 0.4 | 18.0 ± 0.3 |
| Platelets (K/μL) | 823 ± 66 | 826 ± 45 |
| Mean Platelet Volume (fL) | 4.6 ± 0.1 | 4.6 ± 0.1 |

Values = mean ± SE (n=5 mice per group); Abbr.: **BWT**, body weight; **ecSOD**, extracellular superoxide dismutase; **ecSOD-Tg**,

extracellular superoxide dismutase transgenic; **MCS**, mainstream cigarette smoke; **WT**, wild type; *, $p < 0.05$ compared with ecSOD-WT based on Bonferroni's or Dunn's post-test; #, $0.05 \leq p \leq 0.10$ compared with WT based on Bonferroni's or Dunn's post-test; †, $p < 0.05$ for 12 vs 6 cigarettes in WT mice based on Dunn's post-test; ‡, $0.05 \leq p \leq 0.10$ for 12 vs 6 cigarettes in ecSOD-Tg mice based on Dunn's post-test.

showed higher levels of leukocytes after exposure to 6 cigarettes compared with levels after exposure to 12 cigarettes (+178%; $p=0.002$ and +185%; $p=0.001$, respectively) (Tables 7.5 & 7.6). Likewise, lymphocytes were higher in both WT (+174%; $p<0.001$) and Tg (+137%; $p=0.001$) mice after exposure to 6 cigarettes than after exposure to 12 cigarettes, while neutrophils (+289%; $p=0.04$) and monocytes (+90%; $p=0.07$) were only higher in Tg mice (Tables 7.5 & 7.6). MCS seemed to induce a greater response mice in erythrocytic measures of Tg mice, with differences noted in red blood cell count (+18%; $p=0.01$) and measures of hemoglobin (+23%; $p=0.01$) and hematocrit (+18%; $p=0.01$) between Tg mice exposed to 12 or 6 cigarettes (Tables 7.5 & 7.6). Acute exposure to MCS (6 or 12 cigarettes) did not affect plasma levels of CK (striated muscle), ALT or AST (liver), or LDH (non-specific cell toxicity) enzymes in either WT or Tg mice (Tables 7.7 & 7.8), but a number of differences were once again noted between mice of the same background exposed to a different number of cigarettes. Cholesterol was modestly higher in both WT (+16%; $p=0.07$) and Tg (+13%; $p=0.08$) mice exposed to 6 cigarettes compared with mice exposed to 12 cigarettes (Tables 7.7 & 7.8); both albumin (WT: +17%, $p=0.02$; Tg: +10%, $p=0.09$) and total protein (WT: +16%, $p=0.01$; Tg: +15%, $p=0.01$) showed this same pattern, while LDL was higher in only Tg mice exposed to 6 cigarettes compared with Tg mice exposed to 12 cigarettes (+16%; $p=0.08$) (Tables 7.7 & 7.8). No differences were seen in levels of Sca-1⁺ cells or CACs regardless of background or exposure (Fig. 7.5A & B).

D. Discussion

Studies have shown MCS to be both a source of ROS (466-470) and a promoter of ROS production (471, 472), and the subsequent development of oxidative stress is one

Table 7.7. Plasma biomarkers in male WT or ecSOD-Tg mice acutely exposed to either air or mainstream cigarette smoke (50% of smoke of MCS; 6 KY Reference 3R4F cigarettes).

| Plasma Measurements | MCS (6 cigs, 4d) | |
|------------------------------|------------------|----------------|
| | ecSOD-WT | ecSOD-Tg |
| Cholesterol (mg/dL) | 85.70 ± 5.02† | 85.55 ± 3.96¶ |
| HDL (mg/dL) | 35.81 ± 2.68 | 37.56 ± 2.79 |
| LDL (mg/dL) | 32.62 ± 1.98 | 32.81 ± 1.87¶ |
| Triglycerides (mg/dL) | 49.70 ± 5.88 | 51.68 ± 2.63 |
| Albumin (g/dL) | 3.17 ± 0.17† | 2.98 ± 0.08¶ |
| Total Protein (g/dL) | 5.26 ± 0.23† | 5.11 ± 0.08¶ |
| ALT (U/I) | 27.58 ± 4.61† | 27.02 ± 2.95¶ |
| AST (U/I) | 63.36 ± 14.19 | 67.20 ± 4.75 |
| CK (U/I) | 260.89 ± 111.04 | 333.57 ± 25.98 |
| LDH (U/I) | 218.80 ± 44.05 | 260.40 ± 30.58 |
| Creatinine (mg/dL) | 0.25 ± 0 | 0.28 ± 0 |

Values = mean ± SE (n=4-6 mice per group); Abbr.: **ecSOD**, extracellular superoxide dismutase; **ecSOD-Tg**, extracellular superoxide dismutase transgenic; **HDL**, high-density lipoprotein; **LDL**, low-density lipoprotein; **MCS**, mainstream cigarette smoke; **WT**, wild type; *, p<0.05 compared with WT based on Bonferroni's or Dunn's post-test; #, 0.05≤p≤0.10 compared with WT based on Bonferroni's or Dunn's post-test; †, p<0.05 for 12 vs 6 cigarettes in WT mice based on Dunn's post-test; ¶, 0.05≤p≤0.10 for 12 vs 6 cigarettes in ecSOD-Tg mice based on Dunn's post-test.

Table 7.8. Plasma biomarkers in male ecSOD-WT or ecSOD-Tg mice acutely exposed to either air or mainstream cigarette smoke (MCS; 50% of smoke of 12 KY Reference 3R4F cigarettes).

| Plasma Measurements | MCS (12 cigs, 4d) | |
|------------------------------|-------------------|----------------|
| | ecSOD-WT | ecSOD-Tg |
| Cholesterol (mg/dL) | 73.91 ± 3.20 | 75.42 ± 4.09 |
| HDL (mg/dL) | 33.10 ± 1.32 | 33.15 ± 1.84 |
| LDL (mg/dL) | 28.93 ± 1.29 | 28.33 ± 1.83 |
| Triglycerides (mg/dL) | 41.86 ± 8.75 | 38.89 ± 3.44 |
| Albumin (g/dL) | 2.72 ± 0.10 | 2.71 ± 0.11 |
| Total Protein (g/dL) | 4.54 ± 0.16 | 4.43 ± 0.18 |
| ALT (U/I) | 17.03 ± 1.19 | 19.95 ± 0.69 |
| AST (U/I) | 49.35 ± 4.89 | 55.83 ± 4.82 |
| CK (U/I) | 201.59 ± 29.92 | 252.97 ± 39.54 |
| LDH (U/I) | 178.21 ± 16.26 | 202.75 ± 19.31 |
| Creatinine (mg/dL) | 0.27 ± 0.02 | 0.28 ± 0.03 |

Values = mean ± SE (n=4-6 mice per group); Abbr.: **ecSOD**, extracellular superoxide dismutase; **ecSOD-Tg**, extracellular superoxide dismutase transgenic; **HDL**, high-density lipoprotein; **LDL**, low-density lipoprotein; **MCS**, mainstream cigarette smoke; **WT**, wild type; *, p<0.05 compared with WT based on Bonferroni's or Dunn's post-test; #, 0.05≤p≤0.10 compared with WT based on Bonferroni's or Dunn's post-test; †, p<0.05 for 12 vs 6 cigarettes in WT mice based on Dunn's post-test; ‡, 0.05≤p≤0.10 for 12 vs 6 cigarettes in ecSOD-Tg mice based on Dunn's post-test.

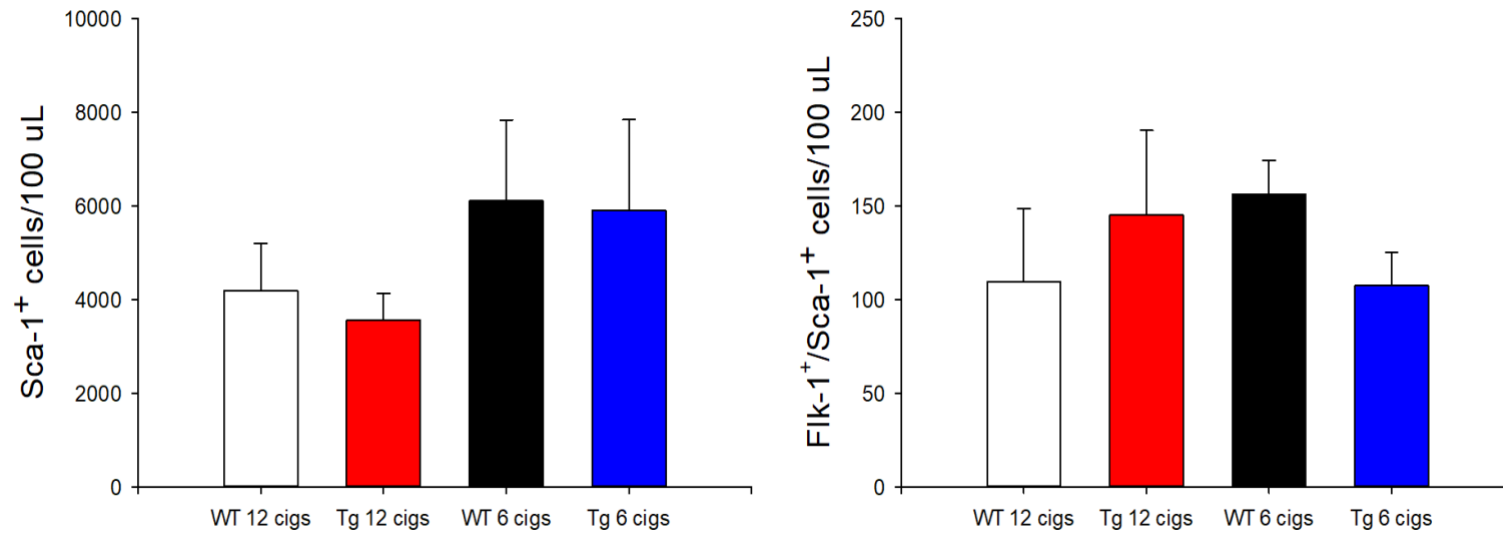


Fig. 7.4. Effects of acute mainstream cigarette smoke (MCS; 6 or 12 cigarettes) exposure on circulating stem cells in male WT and ecSOD-Tg mice. Hematopoietic stem cells (Sca-1⁺) and circulating angiogenic cells (CACs) were measured by flow cytometry in male WT and ecSOD-Tg male mice exposed to HEPA-filtered air or MCS (6 or 12 cigs; 6h/d) for 4 days. After 4 days of exposure to MCS, there were no changes in the level of Sca-1⁺ cells (**A**) or CACs (**B**) regardless of background or the number of cigarettes. Values = mean \pm SE (n=4-6 mice per group).

mechanism through which MCS contributes to the development of CVD (467, 471-474). The SOD enzyme serves to dismutate the superoxide radical and thus works to attenuate the development of oxidative stress in the vasculature. Studies of both under- and overexpression of ecSOD have demonstrated the role of this enzyme in the development of vascular changes and injury that could contribute to CVD and other smoking-related diseases (299, 475-478). The TRPA1 channel is a non-selective, cation channel and a promiscuous receptor concentrated in sensory fibers that mediates pain reception/transmission as well as irritant-related pulmonary reflexes including cough and bronchoconstriction in response to unsaturated aldehydes generated in MCS (261). TRPA1 can also be activated by ROS (263-266, 461, 462), which may also contribute to the channel's role in the development of CVD. The goal of the current studies was to explore the roles of ecSOD and the TRPA1 channel through the use of transgenic (ecSOD-Tg mice and TRPA1-null) mice in response to MCS and CR exposures.

Male ecSOD-Tg mice and their WT littermates were exposed to MCS generated from 6 or 12 KY Reference 3R4F cigarettes. Based on the overall data, there was no discernable indication that ecSOD-Tg mice were better protected from MCS-generated aldehydes than their similarly-exposed WT counterparts through increased levels of pulmonary ecSOD. Urinary measures of acrolein and crotonaldehyde metabolites showed no significant differences between WT and Tg mice exposed to either 6 or 12 cigarettes. There were also no significant differences in measures of urinary nicotine between WT and Tg mice, and despite one set of mice being exposed to smoke from twice as many cigarettes, levels of nicotine were similar between the two exposures in both strains of mice.

Measures of systemic injury assessed through CBC and measures of plasma biomarkers showed minimal differences between similarly-exposed WT and Tg mice, but there were a number of significant differences in both WT and Tg mice between the two exposures to a different number of cigarettes. Mice exposed to 6 cigarettes had higher leukocyte counts, with WT mice having a higher level of lymphocytes and Tg mice having higher levels of neutrophils, lymphocytes, and monocytes compared with mice exposed to 12 cigarettes. Likewise, both WT and Tg mice exposed to 6 cigarettes had higher levels of cholesterol, albumin, total protein, and ALT compared with mice exposed to 12 cigarettes. Measures of CACs also show that levels of these cells were not significantly different between WT and Tg mice exposed to the same number of cigarettes.

These data demonstrate increased systemic and hematological changes with a greater concentration of MCS exposure despite similar levels of urinary nicotine but do not seem to indicate that pulmonary overexpression of ecSOD is protective against injury related to MCS-exposure. Despite studies linking ROS with cigarettes and the development of CVD, it may be that ROS other than superoxide are responsible for this development. However, studies examining SOD in the role of smoking-related pulmonary disease have found that the overexpression of SOD can attenuate pulmonary inflammation and impaired lung function as well as the development of emphysema (477, 478), implicating superoxide in the development of pulmonary diseases related to the use of cigarettes. Other data demonstrate the role of ecSOD in the PM_{2.5}-related impact on levels of CACs (299), and while PM is a known component of cigarette smoke (81), it may be that other constituents of MCS have a counteractive effect that, while potentially not injurious, is not easily visible in MCS as a complete mixture. Additionally, it could be that

the adverse effects noted in response to MCS exposure are induced by systemic ROS, and thus the overexpression of ecSOD specifically in the lungs would not have an effect. It has been shown via measures of protein-acrolein adducts in the plasma and bone marrow that inhaled acrolein, a major constituent of MCS, is able to move into the bloodstream and exert its effects on CACs directly rather than indirectly by solely causing effects via the lungs (74). Although there is some endogenous production of acrolein and thus some naturally-occurring protein-acrolein adducts, the significantly increased levels of these adducts in acrolein-exposed mice indicates delivery of acrolein from the lungs to systemic sites where it can induce adverse effects (74). Based on these data, it may be systemic ROS is more important to cardiovascular-related outcomes than those found in the lungs; use of mice overexpressing ecSOD outside of the lungs or ecSOD knock-out mice may provide additional data regarding the role of ROS in the MCS-induced cardiovascular effects. Additionally, the use of ecSOD-Tg mice in chronic exposures and in exposures to specific constituents such as aldehydes could help clarify the role of superoxide in CVD and other diseases related to tobacco-product use and could help also identify which constituent(s) is responsible for specific outcomes.

The role of TRPA1 in the cardiovascular response to MCS and a constituent unsaturated aldehyde was also explored. Male TRPA1-null mice acutely exposed to MCS (4d; 12 cigarettes) showed minimal systemic changes compared with air-exposed control mice, the most significant being a decrease in leukocytes driven by decreases in lymphocytes and monocytes. However, this leukopenia was also noted in WT mice exposed to MCS under the same conditions, which would indicate that this effect is not due to activation of TRPA1. Likewise, the minimal systemic effects noted in both WT and

TRPA1-null mice after MCS exposure would seem to indicate that the TRPA1 receptor is not serving a significant role in regard to these measures. Additionally, although MCS-exposed WT mice showed a significant decrease in Sca-1⁺ cells, neither Sca-1⁺ cells nor CACs were measured in MCS-exposed TRPA1-null mice, so the impact of this receptor on these cells cannot be determined from the current study.

The lack of significant differences between WT and TRPA1-null mice exposed to MCS suggest that the outcomes measured in the current study are not mediated by TRPA1. However, other studies have clearly demonstrated the role of TRPA1 in MCS-induced diseases (261, 288, 479, 480), which may indicate that non-cardiovascular effects (e.g., pulmonary effects) are the most sensitive outcomes of MCS-exposure. The differing data may also indicate that our acute (4d) exposure was not long enough to induce significant cardiovascular effects detectable in the measured outcomes or that 50% of the smoke from 12 KY Reference 3R4F cigarettes was not sufficient to produce a significant cardiovascular-related effect.

To test whether the effects of CR, an unsaturated aldehyde found in high levels in MCS (156, 159), were also mediated by TRPA1, we exposed male TRPA1-null mice to CR (4d; 1 ppm). In contrast to the effects observed in CR-exposed male WT mice, TRPA1-null mice had no systemic changes in CBC, and plasma levels of the majority of the measured biomarkers were unchanged between the control and CR-exposed TRPA1-null mice. Cholesterol was decreased by CR exposure in the TRPA1-null mice (a change also observed in WT mice), although this change was due to decreased levels of LDL rather than by HDL as observed in WT mice. Furthermore, although it was only modestly significant, TRPA1-null male mice acutely exposed to CR showed an increase in CACs,

whereas WT mice showed no change. Although some of the changes seen in WT and TRPA1-null mice in response to CR exposure are similar, the data overall—including data from other outcomes measured in CR-exposed WT and TRPA1-null mice (481, 482)—seem to indicate that the vascular responses observed in male WT mice following CR exposure were mediated by TRPA1 activation, although chronic studies using TRPA1-null mice would serve to confirm this finding.

In summary, the studies presented in this chapter examined the roles of SOD and TRPA1 in the mechanism through which MCS and CR, an unsaturated aldehyde constituent, contribute to the development of CVD. Although previous studies have shown a role of superoxide and SOD in the development of smoking-related pulmonary diseases and in the mechanism through which PM_{2.5} decreases levels of CACs, the current study did not demonstrate a protective effect due to overexpression of pulmonary ecSOD in the systemic, hematological, and endothelium-related changes related to MCS. Chronic studies could help clarify whether protection conveyed by ecSOD occurs with longer exposure to MCS, while studies exposing ecSOD mice to 100% of the smoke generated from 6 or 12 cigarettes would help determine whether the lack of significant data was a result of levels of exposure too low to induce systemic or vascular changes. Additionally, exposure to specific constituent aldehydes could help identify whether individual components cause cardiovascular injury that may be masked by MCS as a complete mixture. Although the current study did not seem to suggest that the hematological, systemic, and CAC-related effects of MCS are produced through TRPA1, the current study of TRPA1-null mice exposed to CR does support the hypothesis that the cardiovascular effects of unsaturated aldehydes are mediated by TRPA1. Again, chronic studies of

TRPA1-null mice exposed to MCS and to CR would further strengthen these findings.

Because of the commonality of exposure to MCS and unsaturated aldehydes from everyday sources and because of the risk of high-level aldehyde exposures related to accidents, chemical attacks, or occupational exposures, a better understanding of the mechanisms through which these compounds lead to CVD is necessary. These studies present data on the role of SOD in MCS-induced systemic injury and present novel research implicating TRPA1 in the mechanism of CR-induced toxicity, strengthening the hypothesis that CR works through a mechanism similar to acrolein to induce its cardiovascular effects.

CHAPTER VIII

SUMMARY & CONCLUSIONS

A. Major Findings

1. Louisville Healthy Heart Study

Circulating angiogenic cells (CACs) have been shown to be associated with endothelium health (62-66) as well as with well-known cardiovascular disease (CVD) risk factors (66, 70, 305-308). CACs have also been associated with smoking, both with mainstream cigarette smoke (MCS) (315-319) and with individual MCS constituents, particularly acrolein (71, 74, 151). Although the health effects of e-cigarettes (e-cigs) are still largely unknown, these shared constituents may contribute to adverse cardiovascular effects similar to those seen with the use of traditional cigarettes. Thus, the Louisville Healthy Heart Study was designed to examine the effect of tobacco product use (never smoker, former smoker, cigarette only use, and e-cig or dual use) on hematological measures, measures of urinary VOC and tobacco alkaloid metabolites, and levels of CACs.

E-cig or dual users were significantly younger than other product use categories, an outcome similar to other studies that have found that e-cig use is most common among youth and young adults, but had significantly higher levels of leukocytes. Few studies have examined the effects of e-cig use on hematological outcomes, but the increased levels of

leukocytes previously seen in smokers (345, 351-355) seem to indicate that there may be a similar mechanism inducing this increase. As this increase in smokers is typically attributed to inflammation (345, 354, 356-358), additional measures of inflammatory markers in the LHHS cohort could help clarify this relationship. Analysis of urinary VOC metabolites showed significantly higher levels in users of tobacco products compared with nonsmokers. Although there were several metabolites that were significantly higher in cigarette only smokers compared with e-cig or dual users—supporting other studies showing that the production of VOCs in e-cigs may be less than that of traditional cigarettes—the levels measured in e-cig or dual users were still significantly higher than levels in both never and former smokers, indicating that the use of e-cigs alone or in combination with traditional cigarette retains the potential to induce cardiovascular effects through increased levels of VOCs. Analysis of CACs between the tobacco product use categories showed that only e-cig or dual users had levels of CACs that were significantly different than never smokers. CAC-3 (CD31⁺/CD34⁺/AC133⁺/CD45^{dim}), an early endothelial progenitor cell population was significantly increased in e-cig or dual users, potentially indicative of increase endothelium repair initiated by a low but persistent level of injury induced by e-cig use. CAC-12 (CD45⁺), however, was significantly decreased with e-cig or dual use; more research is needed to clarify the role of the CD45 antigen in CACs.

Additional studies in a larger cohort and/or in a cohort with a greater number of e-cig only users are needed to strengthen these findings. However, the similarity of outcomes seen between e-cig and dual users and users of traditional cigarettes alone seem to suggest that e-cig are capable of inducing hematological and endothelium-related effects that can

subsequently contribute to the development of CVD. As e-cig alone and dual use becomes more prevalent among individuals of all age groups, it is imperative to gain a better understand of both the acute and chronic cardiovascular effects induced by these emerging tobacco devices.

2. Constituent Aldehyde Exposures and Systemic and Hematological Responses

Tobacco smoking is the most significant modifiable risk factor in the development of CVD (1, 14, 75-77). Exposure to mainstream cigarette smoke (MCS) and individual aldehyde constituents including acrolein, crotonaldehyde (CR), formaldehyde (FR), and acetaldehyde (AA) have been linked to negative cardiovascular health outcomes (1, 91-94). The cardiovascular effects of emerging tobacco products including e-cigs are still largely unknown, although studies have detected aldehydes in e-cig aerosols (96, 180, 207-213); the presence of these aldehydes may indicate the potential for negative cardiovascular effects similar to those seen with MCS. These studies focused on the systemic and hematological changes induced by inhalation exposure to MCS, e-cig aerosol, e-cig humectants, or constituent aldehydes (FR, AA, acrolein, and CR).

Many of our studies of mixtures, humectants, and individual aldehydes showed decreases in leukocytes after acute (4d or 2 weeks) exposure. Specifically, acute (4d) exposures to MCS, JUUL Mango and blu Plus+ aerosols, 30%:70% PG:VG, and acrolein and two weeks of exposure to formaldehyde (5 ppm) caused significant decreases in leukocytes, while the remaining studies showed no significant changes between the air-exposed control and the pollutant-exposed mice. Epidemiological studies of tobacco smokers have shown significant changes in hematological factors, particularly increased

levels of leukocytes (345, 351-355), red blood cells (345, 414), and hemoglobin and platelets (345, 355, 411-413). This contradiction may be related to the use of naïve mice, which may serve as a better model of health effects in new smokers rather than chronic smokers, or to our short exposure durations, which would not necessarily be comparable to effects seen in chronic smokers. Nonetheless, the significance of the changes seen in leukocytes reveal the potential of these mixtures and constituents to promote adverse hematological changes that could contribute to downstream disease development.

Systemically, mice exposed to CR (1 ppm) for 12 weeks showed significant increases in cholesterol and triglycerides; high levels of both of these biomarkers are known risk factors of ischemic (455) and coronary heart disease (456), and their increase in response to chronic CR (1 ppm) exposure may indicate that CR plays a role in these CVDs when MCS is a contributing factor. However, levels of these biomarkers did not increase with acute (4d; 1 ppm) exposure, indicating that increases in lipids are not the primary change initiated by CR exposure.

Our studies of individual constituent aldehydes revealed that the concentration of the saturated aldehyde FR needed to induce significant systemic and hematological changes was much higher than the concentration required to see significant changes after exposure to unsaturated aldehydes (5 ppm versus 1 ppm). The carbon-carbon double bond in unsaturated aldehydes gives them a higher reactivity than saturated aldehydes, which may account for this difference. Acrolein has been shown to be a more potent irritant than FR (483), and odor detection and/or irritation of CR can occur at <1 ppm in approximately 1 minute (155). Chronic exposures to a low level (1 ppm) of FR or AA would allow for the examination of potential differences in cardiovascular toxicity related to saturated

versus unsaturated aldehyde exposures.

3. Aldehyde Exposures and Circulating Angiogenic Cells

CACs are a population of stem cells that serve to repair endothelium dysfunction (62, 63) and that play an important role in overall endothelial function and health (63-66). Levels and function of CACs have been found to be inversely correlated with risk factors for atherosclerosis (70) as well as an individual's Framingham Risk Score (66). Inhalation exposure to acrolein has been shown to decrease levels of CACs in both humans (71) and mice (74, 151), but the effect of mixtures and other constituent aldehydes on CACs are still relatively unexplored. These studies focused on examining changes in CACs in response to inhalation exposures in mice as a marker of potential endothelium dysfunction. Data from these studies revealed that only female mice acutely (4d) exposed to JUUL Mango aerosol and male mice exposed to FR (2 weeks; 5 ppm) showed significant decreases in CACs. Measures of urinary metabolites in JUUL Mango-exposed male mice (6h) showed high levels of 3HPMA, the primary urinary metabolite of acrolein; as acrolein has been shown to decrease blood levels of CACs, it is likely that the decreased levels of CACs noted in JUUL Mango-exposed females is due to acrolein exposure. Additional studies in male mice exposed to JUUL Mango as well as examination of levels of Sca-1⁺ cells, the stem cell population from which CACs are derived, and levels of CACs in the bone marrow would help further clarify the mechanism through which JUUL Mango is inducing the significant decrease in CACs.

Although acute (4d) exposure to 5 ppm FR increased levels of CACs, two weeks of exposure to the same concentration of FR significantly decreased CACs. The increase noted after four days may indicate a compensatory effect to repair damage within the

endothelium, with the subsequent decrease a sign that this compensatory effect cannot be sustained. Urinary measures of formate in male mice exposed to FR alone (5 ppm, 6h) showed similar levels as male mice exposed to MCS (50% smoke of 12 KY 3R4F cigarettes, 6h). Taken with the outcomes from our two-week FR exposure in mice, these data may indicate that FR is contributing at least in part to the endothelium dysfunction noted in chronic smokers (85). However, *in vitro* studies exposing the aorta and the superior mesenteric artery to FR did not induce endothelial dysfunction (127), so the clinical relevance of these changes in CACs remains unclear. Likewise, although acrolein has been linked to endothelium dysfunction in mice (150), neither acute (4d) nor chronic (12 weeks) exposure to CR induced significant endothelium dysfunction (481, 482), nor were significant changes in CACs noted in either male (1 or 3 ppm, 4d; 1 ppm, 12 weeks) or female (1 ppm, 4d) mice with acute exposure. This difference may indicate that acrolein is more toxic to the endothelium than CR, at least at the levels tested. Taken together, it appears that, at the concentrations and durations of exposure used in the current studies, CACs are likely not the earliest biomarker of harm affected in response to FR, AA, or CR exposure. While changes in the levels of these cells may play an important role in vascular and cardiovascular health, these data suggest that they should not be used as the only indicator of cardiovascular changes induced by aldehyde exposure. It would seem more appropriate that the level of CACs taken in conjunction with changes in systemic and hematological biomarkers as well as with measures of vascular function could denote exposure-related vascular changes that could indicate the potential for the development of CVD. Nonetheless, more studies in both male and female mice are needed to better understand the impact of specific constituent aldehydes on CACs and on other measures

of endothelium dysfunction and to gain a better understanding of how these aldehydes contribute to MCS- and e-cig aerosol-induced CVD.

4. Aldehyde Exposures and Sex Differences

The data from our mixtures and individual aldehyde studies are largely consistent with data from a previous study showing that female mice exposed to a high level (250 ppm) of acrolein had reduced morbidity and mortality compared with similarly-exposed male mice (269). Our studies seem to demonstrate that females are protected against FR-induced systemic and hematological changes as well as changes in CACs. However, as most of the changes induced by FR in males occurred after two weeks, a two-week exposure in females at the same concentration (5 ppm) would better clarify this dependence. Additionally, chronic studies in both males and females would provide an even greater understanding of this relationship. Likewise, a chronic exposure of female mice to CR (1 ppm, 12 weeks) would allow for a greater understanding of the sex-dependence noted between male and female mice after acute (4d) exposure to CR (1 ppm). Although male mice acutely exposed to CR showed minimal but significant systemic and hematological changes as well as a significant increase in Sca-1+ cells (1 ppm), females showed no significant changes in any of the measured biomarkers. These differences seem to support the hypothesis that females are somewhat protected against toxicity and injury induced by unsaturated aldehydes.

Several epidemiological studies seem to show outcomes consistent with this hypothesis as well, particularly showing that females are less sensitive to tobacco-induced atherosclerotic damage than males (484); this difference is likely due to the protective effects of female hormones such as estrogen (484). The hormone prolactin may also play

a significant role in the sex-dependent role of these aldehydes. Female mice have been shown to have a higher sensitivity to pain-inducing stimuli than males, driven largely by levels of prolactin (485). Prolactin has been shown to regulate expression of the TRPA1 receptor, which serves as a pain and irritant receptor in sensory neurons (486), and Conklin et al. (269) have proposed that perhaps females have a greater expression of TRPA1 that promotes a quicker response to irritants such as unsaturated aldehydes. In fact, females exposed to high-level acrolein (250 ppm) showed a quicker “respiratory braking” response and decrease their breathing rate quicker than male mice under the same exposure conditions (269). This variance in the change in breathing rate is not seen in male and female TRPA1-null mice, further suggesting that the female protection is related to TRPA1 (277). Additional studies are needed to determine the exact mechanism of this sex-dependent protection in response to exposure to unsaturated aldehydes as well as to mixtures such as MCS and e-cig aerosol.

5. Mainstream Cigarette Smoke and Superoxide Dismutase

The superoxide dismutase (SOD) enzyme indirectly plays a role in maintaining proper endothelium function by catalyzing the dismutation of superoxide. If left as a free molecule in the blood, superoxide can bind with nitric oxide (NO) and decrease the bioavailability of NO (40, 41, 49, 50, 53); the resulting decrease of this vasodilator can allow for the development of endothelium dysfunction (40, 41), which has been linked to a number of CVDs (54-58). SOD has also been linked to the development of cigarette smoke-induced pulmonary diseases (477, 478), and the administration of SOD to serve as an antioxidant defense against the loss of NO—and the subsequent development of endothelium dysfunction and, perhaps, disease—has been proposed (53, 487, 488).

The results of our studies in mice overexpressing pulmonary extracellular SOD (ecSOD-Tg) after exposure to MCS (50% of smoke of 6 or 12 KY Reference 3R4F cigarettes), however, do not seem to support this proposed medical intervention. Although there were differences between WT and ecSOD-Tg mice exposed to cigarettes compared with baseline, there were no significant differences between WT and ecSOD-Tg mice exposed to the same number of cigarettes. Measures of urinary metabolites did not show significant differences in the excretion of unsaturated aldehyde metabolites or total urinary nicotine metabolites between the two strains of mice, and measures of systemic and hematological biomarkers showed few if any significant differences between WT and ecSOD-Tg mice at the same level of exposure. Likewise, CAC levels were not significantly different between the two strains after exposure to either 6 or 12 cigarettes. Chronic studies would help elucidate whether any protective effect of SOD exists but takes longer than four days to become apparent in the measured outcomes. Additionally, exposures of ecSOD-Tg mice to individual constituent aldehydes could help explore the role of SOD in the mechanism of specific aldehydes.

6. Aldehydes and the Transient Receptor Potential Ankyrin 1 Channel

The transient receptor potential ankyrin 1 (TRPA1) channel is activated by a variety of air pollutants, including MCS and aldehydes (127, 260, 262, 266, 268-270). Although TRPA1 is primarily recognized as a pain receptor (262, 272, 279), it also play an important role in the irritant response induced by inhaled toxins (262, 264, 279, 286), including respiratory braking (slowing rate and depth of breathing), which serves to reduce inhalation and thus exposure to the toxin (261, 489). This function is supported by the delayed onset of respiratory braking and the higher incidence of morbidity and mortality seen in TRPA1-

null mice exposed to a high level of acrolein compared with similarly-exposed WT mice (269). The limited changes induced by CR in TRPA1-null mice compared with CR-exposed WT mice seem to indicate that the systemic and hematological changes noted in male WT mice following CR exposure were mediated by TRPA1. Chronic studies using TRPA1-null mice would serve to strengthen this finding, while a chronic study exposing TRPA1-null mice to acrolein under the same conditions (1 ppm; 12 weeks) would further confirm the hypothesis that unsaturated aldehydes induce CVD effects through the TRPA1 channel. Additionally, exposures of TRPA1-null mice to FR and AA would allow for the determination as to whether saturated aldehydes induce CVD effects through the same pathway. Studies have shown that the pain responses induced by FR and AA are mediated through TRPA1 (490, 491) as is the FR-induced—but not the AA-induced—relaxation of the superior mesenteric artery (127, 492), but the mechanism through which these aldehydes induce cardiovascular effects has not been determined.

B. Strengths

There are several strengths of the human study presented in this dissertation. First, although the Louisville Healthy Heart cohort was not as large as national epidemiological cohorts, we were still able to examine tobacco product use, urinary VOC metabolites, and CACs in a population of up to 143 individuals. Many previous investigations of CVD and CACs (66, 70, 307, 309, 493) as well as several studies that have looked at CACs in relation to smoking (315, 316, 318) have utilized cohorts of less than 100 people, while only a few studies (311, 494, 495), including the predecessor to our study (71) have examined these relationships in larger cohorts. Additionally, two-thirds of our participants report a diagnosis of some form of CVD, and as the prevalence of CVD continues to grow

worldwide, it becomes increasingly important to perform research in susceptible individuals to provide accurate data that can be used to inform the development of better cardiovascular health care practices. Our study is also one of the first to look at the relationship between CACs and the use of ENDS devices, especially in fifteen antigenically distinct CAC populations. Looking at the association between these populations, product use, and CVD allowed us to examine changes in specific cell types and subpopulations that may be linked to specific risk factors, as had previously been shown (73).

There are also a number of strengths in our murine exposure studies. The results of our urinary metabolite studies demonstrate that the levels of exposure used in our individual constituent aldehyde studies are comparable to real-world exposures, which validates our outcomes as cardiovascular and systemic effects that could be seen in humans in response to aldehyde exposures. Our studies of CR are the first to show the systemic, hematological, and endothelium-related effects of CR exposure *in vivo* and to further show that these effects are both sex- and TRPA1-dependent, the latter of which may present a therapeutic target for treatment of aldehyde exposures. Our studies of FR and AA are also some of the first to look at cardiovascular effects in response to acute exposures and again to suggest sex-dependent differences in outcomes.

C. Limitations

Despite the many strengths of our studies, there are also a number of limitations. Despite the size of the Louisville Healthy Heart cohort compared with other cohorts examining CACs, the study is still relatively small, and it would therefore be unwise to extrapolate the findings to the population at large, although the outcomes do present interesting and specific effects that ought to be examined in a larger cohort. Likewise, the

number of e-cig only users and dual users was small. Other cohorts report similar limitations in recruiting e-cig-only users (395), and large epidemiological studies have reported that e-cig users are likely to be dual users (496, 497), making recruitment of e-cig only users more difficult. However, if dual use is the most common form of e-cig use, having a dual use group in future cohort studies would allow for analysis of outcomes in a group most relevant to real-world e-cig use. Of the e-cig alone and dual users in our study, all reported use of 1st and 2nd generation e-cigs (cigalikes and vape pens, respectively), and thus our findings cannot be generalized to users of 3rd (box mod) and 4th (vape pod) generation e-cig devices or to more recent products such as puff pods and puff bars. The self-report nature of our questionnaire also presents a limitation, especially in regarding to self-reported smoking status. Although questions related to tobacco use or exposure to secondhand smoke were carefully structured to avoid misclassification, we can only assume the individuals were truthful in their responses. We can assume some level of confidence in self-reported smoking status based on measured of urinary cotinine, but non-smoking individuals who are exposed to high levels of secondhand smoke could have relatively high levels of urinary cotinine, which could confound the outcomes.

In regard to other measures that could play an important role in the development of CVD, although we have measures of body mass index (BMI) for the participants, we do not have measures of visceral adiposity or waist-to-hip ratios. These alternate measures have been proposed as comparable if not better indicators of obesity than BMI (498), and several epidemiological studies have found significant associations between waist-to-hip ratios and cardiovascular events and/or cardiovascular-related death (499-501). As obesity is a known risk factor for CVD (502, 503), future human studies examining CVD should

make obtaining these measures part of their protocol in order to provide a more comprehensive assessment of a participant's CVD risk.

There are also limitations in our murine studies. First, mice are “involuntary smokers,” and they therefore decrease the rate and depth of their breathing in response to exposure to noxious compounds such as aldehydes (269); this reflex is known as the “respiratory braking response” (489). Second, estimations of actual aldehyde exposure in mice was measured by urinary metabolites. Because aldehydes metabolize quickly (99, 100, 108, 109, 504), it is difficult to measure levels of these compounds in the blood, but levels of urinary metabolites have been shown to be proportional to and thus useful biomarkers of aldehyde exposure (147, 159). Nonetheless, even at relevant exposure concentrations, our studies did not always show significant systemic or hematological changes. This could be because the level of exposure was too low and/or the duration of the exposure was not sufficient to cause significant cardiovascular outcomes. The duration of our exposure is also a limitation in that the time frame during which mice are exposed (6h/day) may be more concentrated than that of a typical tobacco product user. That is, although the puff protocol used in our MCS and e-cig exposures are designed to mimic the episodic nature of tobacco product use (smoking or vaping for a brief session followed by a period of non-use before smoking or vaping again), tobacco product use in humans does not occur only within a six hour window in any given day; the longer window of exposure would likely allow for variations in metabolism and clearance greater than that seen in our urinary metabolite data and, thus, likely allow for a lower level of exposure to harmful constituents. Therefore, although the effects induced in our exposed mice may indicate effects that could occur in tobacco product users, these outcomes may take longer to

manifest in humans compared with mice when accounting for differences in daily exposure durations. The aldehydes used in our studies are known to be carcinogenic, and it is possible that cancer would develop before the development of negative cardiovascular effects at the tested concentrations and durations. Additionally, CACs as a marker of vascular injury were not always measured due to limited blood available for analyses, so it is possible changes in CACs that could lead to subsequent CVD development were induced but not noted. Similarly, we did not always perform the same exposures in both male and female mice, so we cannot necessarily speak to the sex-dependence of the measured outcomes when there was not a directly comparable study.

D. Future Directions

More research is still needed to explore the toxicity of traditional cigarettes and ENDS in both human cohorts and murine models. Additional studies with focused recruitment of e-cig only users are needed in order to better understand the health effects attributable to e-cig use rather than effects potentially compounded from the use of both e-cigs and traditional cigarettes. These cohorts should also work to recruit users of more recent generations of e-cig devices (mechanical mods, variable voltage devices, JUUL and other pod-based devices) and other ENDS to better understand differences in health outcomes related to different ENDS, especially as more recent generations of these devices continue to grow in popularity among adolescents. In regard to research using murine models, future studies could increase the concentration of the aldehydes used for exposure—although concentrations should be compared with those found in mixtures to ensure their real-world relevance—or the length of the exposure to test whether chronic exposures at varying doses, as would be relevant in addicted tobacco-product users, produce additional

systemic and vascular changes.

Furthermore, despite looking at a number of different aldehydes, the actual constituent responsible for the toxicity of smoking has not been identified, so additional studies of different shared constituents as well as additional e-cig flavorings could help with this identification. Combination studies should also be performed using individual aldehyde constituents and nicotine. As users of tobacco products are exposed to aldehydes and nicotine simultaneously, and as nicotine alone has been shown to induce cardiovascular effects, a better understanding of how aldehydes and nicotine in combination affect our cardiovascular outcomes of interest is required, particularly if exposure to these compounds in combination could potentially induce outcomes varying from those seen with individual exposures. Finally, as we used whole-body TRPA1-null mice in our MCS and unsaturated aldehyde studies, we cannot rule out a role of extra-pulmonary (neuronal) TRPA1 in these outcomes. In fact, TRPA1 receptors are widely distributed in non-neuronal sites, such as the urothelium, the heart, and the vasculature including the endothelium (127, 275-277, 280). The exact location of the TRPA1 that is activated by inhaled MCS and MCS-related toxicants (and responsible for systemic effects) will need to be established in more specific models of TRPA1 deletion.

E. Conclusions

Overall, the work described here demonstrates the adverse systemic, hematological, and endothelium-related effects induced by exposure to traditional cigarettes and e-cigs and supports the hypothesis that much of the cardiovascular injury related to the use of these products is related to aldehydes, particularly the unsaturated aldehydes, through the TRPA1 channel.

Our human studies showed that e-cig or dual users were younger than other tobacco product use categories, as has been reported in previous cohort studies. Relatedly, the e-cig or dual users in our study had significantly less prevalence of CVD and of CVD-related medication usage. E-cig or dual users also showed significantly increased levels of leukocytes—perhaps indicative of increased inflammation as seen in users of traditional cigarettes—as well as increased levels of CAC-3 (CD31⁺/CD34⁺/AC133⁺/CD45^{dim}), which may be related to a persistent, low level of injury induced by some unique component of e-cigs or of combined e-cig and cigarette use. Conversely, CAC-12 (CD45⁺) was significantly decreased in this group of users, but further study is needed to better understand this change. Although e-cig or dual users had levels of some urinary VOC metabolites that were significantly lower than levels in users of traditional cigarettes alone, almost all measured VOC metabolite levels were still significantly higher in e-cig or dual users compared with both never and former smokers. These data confirm that while e-cigs may be less harmful than traditional cigarettes in regard to VOC exposures, they still produce VOCs at levels capable of inducing adverse cardiovascular effects.

Our murine studies indicate that short-term exposures to e-cig aerosols and PG:VG produce hematological changes similar to those seen with MCS exposure, as does two weeks of exposure to FR. Similarly, chronic CR exposure shows dyslipidemic changes that could contribute to the development of CVD. Short-term exposures to AA, however, produced limited systemic and hematological changes. Only short-term exposure to JUUL Mango in females and two weeks of exposure to FR (5 ppm) causes significant decreases in CACs, indicating that endothelium dysfunction is likely not one of the early changes caused by exposure to these mixtures and individual aldehydes, at least not at the

concentrations and durations tested. The specificity of the change in CACs does also indicate the need for additional studies of specific e-cig flavors, as different flavors may cause different cardiovascular outcomes, and for the continued study of individual aldehyde constituents. Additionally, these studies show the importance of the TRPA1 receptor in the systemic and hematological changes induced by CR exposure as well as the sex-dependent nature of CR and, potentially, FR and e-cig aerosols.

Future studies are required to better understand the role of tobacco products, particularly e-cigs, in the development of CVD and to continue working towards a better understanding of the mechanisms through which aldehydes contribute to the burden of tobacco-related CVD.

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ABBREVIATIONS

| | |
|-------|--|
| AA | Acetaldehyde |
| AAMA | N-acetyl-S-(2-carbamoylethyl)-L-cysteine |
| ACE | Angiotensin-converting enzyme |
| ALDH2 | Aldehyde dehydrogenase |
| ALT | Alanine aminotransferase |
| AMCC | N-acetyl-S-(N-methylcarbamoyl)-L-cysteine |
| ANB | Anabasine |
| ANOVA | Analysis of variance |
| ANTB | Anatabine |
| AST | Aspartate aminotransferase |
| BMA | N-acetyl-S-(benzyl)-L-cysteine |
| BSA | Bovine serum albumin |
| BWT | Body weight |
| CABG | Coronary artery bypass grafting |
| CAC | Circulating angiogenic cell |
| CBC | Complete blood cell count |
| CDC | Centers for Disease Control and Prevention |
| CEMA | N-acetyl-S-(2-carboxyethyl)-L-cysteine |
| CIG | Cigarette |

| | |
|----------|--|
| CIMT | Carotid intima-media thickness |
| CK | Creatinine kinase |
| COT | Cotinine |
| CR | Crotonaldehyde |
| CVD | Cardiovascular disease |
| DHBMA | N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine |
| e-cig | Electronic cigarette |
| ecSOD | Extracellular superoxide dismutase |
| ecSOD-Tg | Extracellular superoxide dismutase transgenic |
| ENDs | Electronic nicotinic delivery system |
| eNOS | Endothelial nitric oxide synthase |
| EPC | Endothelial progenitor cell |
| EVALI | E-cigarette, or vaping, product use associated lung injury |
| FACS | Florescence-activated cell sorting |
| FR | Formaldehyde |
| GC-MS | Gas chromatography/mass spectrometry |
| 3HC | Trans-3'-hydroxycotinine |
| HDL | High-density lipoprotein |
| HEPA | High-efficiency particulate air |
| 2HPMA | N-acetyl-S-(2-hydroxypropyl)-L-cysteine |
| 3HPMA | 3-hydroxypropylmercapturic acid |
| HPMMA | 3-hydroxy-1-methylpropylmercapturic acid |
| HS | High school |

| | |
|--------|--|
| KY | Kentucky |
| LDH | Lactate dehydrogenase |
| LDL | Low-density lipoprotein |
| LHHS | Louisville Healthy Heart Study |
| MA | Mandelic acid |
| MCS | Mainstream cigarette smoke |
| 2MHA | 2-methylhippuric acid |
| 3MHA | 3-methylhippuric acid |
| 4MHA | 4-methylhippuric acid |
| 3MHBMA | N-acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine |
| NHANES | National Health and Nutrition Examination Survey |
| NIC | Nicotine |
| NO | Nitric oxide |
| O/N | Overnight |
| PBS | Phosphate-buffered saline |
| PCI | Percutaneous coronary intervention |
| PG | Propylene glycol |
| PGA | Phenylglyoxylic acid |
| PM | Particulate matter |
| PMA | Platelet mononuclear cell aggregate |
| RDW | Red blood cell distribution width |
| REDCap | Research Electronic Data Capture |
| RHI | Reactive hyperemia index |

| | |
|------------|--|
| ROS | Reactive oxygen species |
| SES | Socioeconomic status |
| SHS | Secondhand smoke |
| SOD | Superoxide dismutase |
| TG | Transgenic |
| THC | Tetrahydrocannabinol |
| Total NIC | Total nicotine |
| TRPA1 | Transient receptor potential ankyrin 1 |
| TSP | Total suspended particulate |
| UPLC-MS/MS | Ultra-performance liquid chromatography tandem mass spectrometry |
| VA | Virginia |
| VG | Vegetable glycerin |
| VOCs | Volatile organic compounds |
| WT | Wild type |

CURRICULUM VITAE

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EDUCATION

- 2013-2016 M.S. in Pharmacology and Toxicology; University of Louisville, Louisville, KY
- 2011-2013 M.S. in Biological Sciences; Southern Illinois University Edwardsville, Edwardsville, IL
- 2007-2011 B.S. in Biology, Minors in Chemistry and Biochemistry; McKendree University, Lebanon, IL

PROFESSIONAL EXPERIENCE

- 2013-Present Graduate Research Assistant, University of Louisville, Department of Pharmacology and Toxicology
- 2011-2013 Forensic Toxicology Laboratory Staff, Saint Louis University
- 2010 Forensic Toxicology Laboratory Staff, Saint Louis University

PROFESSIONAL MEMBERSHIPS AND ACTIVITIES

- 2016-Present Society of Toxicology, Student Member
- 2016-Present Ohio Valley Society of Toxicology
- 2013 Phi Kappa Phi Honor Society
- 2012-2013 Illinois State Academy of Science

2008-2011 Sigma Zeta National Science and Mathematics Honor Society

HONORS AND AWARDS

2017 Ohio Valley Society of Toxicology Ph.D. Poster Presentation Award
2017 University of Louisville School of Interdisciplinary and Graduate Studies
Travel Award
2017 Society of Toxicology Graduate Student Travel Support Award
2016-2018 NIEHS T32 Training Program in Environmental Health Sciences Pre-
Doctoral Training Grant
2013 Illinois State Academy of Science Environmental Health Science Poster
Presentation Award
2012 Southern Illinois University Edwardsville Research Grant for Graduate
Students
2011 McKendree University Honors Program Graduate
2011 McKendree University Magna Cum laude
2011 McKendree University Biology Department Top Student Award
2009-2011 McKendree University Dean's List
2009-2011 McKendree University Carrie Field Smith and Emma Lee Foree Walkup
Smith Memorial Scholarship
2009-2011 McKendree University Edwin P. Baker Scholarship
2007-2011 McKendree University Founder's Scholarship
2007-2011 McKendree University Dance Team Scholarship
2007-2008 McKendree University President's List

SERVICE

2019-Present Sojourn Church East SojournKids Volunteer
2017-2019 Sojourn Church East Student Ministry Leader

LEADERSHIP

2010-2011 McKendree University Dance Team Captain

2009-2011 Sigma Zeta National Science and Mathematics Honor Society Beta Chapter
 Vice President

ABSTRACT AND PRESENTATIONS

Oral

Sigma Zeta National Science and Mathematics Honors Society National Convention

- What to Do with a Dead Body: A Forensic Evaluation of Skeletal Remains. San Antonio, TX, 2011

Poster

Superfund Research Program Annual Meeting

- **Jordan Finch**#, Stacey Konkle#, Aruni Bhatnagar, Katlyn McGraw, Haley Metcalf, Jamar Wheeler, Lauren Heberle. What Do We Know? A Critical Review of Site Condition and Public Health Risk Assessments in EPA Five-Year Reviews of the Lee's Lane Superfund Site in Louisville, KY. Seattle, WA, 2019. #, co-first authors.

Society of Toxicology Annual Meeting

- **Jordan Finch** and Daniel J. Conklin. Effects of Tobacco Product-Derived Unsaturated Aldehydes on Circulating Angiogenic Cells. Baltimore, MD, 2019.
- **Jordan Finch**, Andre Richardson, Lexiao Jin, Daniel J. Conklin. Effects of Tobacco Product-Derived Aldehydes on Circulating Angiogenic Cells—Implications for Endothelium Dysfunction and Repair. San Antonio, TX, 2018.
- **Jordan Finch**, Gary Hoyle, Connie Schlueter, Ian McKinley, Aruni Bhatnagar, Daniel J. Conklin. Cardiopulmonary Effects of Acute Exposure to Electronic Cigarettes and Formaldehyde in Mice. Baltimore, MD, 2017.

Ohio Valley Society of Toxicology Regional Chapter Annual Meeting

- **Jordan Finch**, Andre Richardson, Pawel Lorkiewicz, Zhengzhi Xie, Daniel J. Conklin. Effect of Tobacco Product-Derived Saturated and Unsaturated Aldehydes on Circulating Angiogenic Cells. Procter & Gamble Mason Business Center, Mason, OH, 2019.

- **Jordan Finch** and Daniel J. Conklin. Effects of Tobacco Product-Derived Unsaturated Aldehydes on Circulating Angiogenic Cells. University of Louisville, Louisville, KY, 2018.
- **Jordan Finch**, Andre Richardson, Lexiao Jin, Daniel J. Conklin. Effects of Tobacco Product-Derived Aldehydes on Circulating Angiogenic Cells—Implications for Endothelium Dysfunction and Repair. Purdue University, West Lafayette, IN, 2017.
- **Jordan Finch**, Gary Hoyle, Connie Schlueter, Ian McKinley, Aruni Bhatnagar, Daniel J. Conklin. Cardiopulmonary Effects of Acute Exposure to Electronic Cigarettes and Formaldehyde in Mice. Eli Lilly Corporate Center, Indianapolis, IN, 2016.

University of Louisville Research!Louisville

- **Jordan Finch**, Andre Richardson, Pawel Lorkiewicz, Zhengzhi Xie, Daniel J. Conklin. Effect of Tobacco Product-Derived Saturated and Unsaturated Aldehydes on Circulating Angiogenic Cells. Louisville, KY, 2019.
- **Jordan Finch**, Andre Richardson, Lexiao Jin, Daniel J. Conklin. Role of Crotonaldehyde in the Cardiovascular Toxicity of Mainstream Cigarette Smoke. Louisville, KY, 2018.
- **Jordan Finch**, Andre Richardson, Daniel J. Conklin. Role of Tobacco Smoke-Derived Aldehydes on Circulating Angiogenic Cells—Implications for Endothelium Repair. Louisville, KY, 2017
- **Jordan Finch**, Gary Hoyle, Connie Schlueter, Aruni Bhatnagar, Daniel J. Conklin. Electronic Cigarette Exposure and Pulmonary Function in Mice: Role of Formaldehyde. Louisville, KY, 2016.
- **Jordan Finch**, Petra Haberzettl, Aruni Bhatnagar, Daniel J. Conklin. Air Pollution, Pulmonary Oxidative Stress, and the Endothelin System in the Development of Cardiovascular Disease. Louisville, KY, 2015.
- **Jordan Finch**, Timothy E. O'Toole, James McCracken, Natasha DeJarnett, Aruni Bhatnagar, C. Arden Pope III, Daniel J. Conklin. Air Pollution and Biomarkers of Cardiovascular Injury -- Endothelin-1 and Endothelial Progenitor Cells in Humans. Louisville, KY, 2014.

University of Louisville Cardiovascular Research Symposium

- **Jordan Finch** and Daniel J. Conklin. Effects of Tobacco Product-Derived Unsaturated Aldehydes on Circulating Angiogenic Cells. Louisville, KY, 2019.

Illinois State Academy of Science Annual Meeting

- **Jordan Finch**, Kurt Schulz, Richard Brugam, Zhi-Qing Lin, Luci Kohn. A Study in Lead: An Analysis of Human Lead Exposure in the Old Lead Belt of Southeastern Missouri. Jacksonville, IL, 2013.

Publications

- **Lynch, J.**, Jin, L., Richardson, A., Jagatheesan, G., Lorkiewicz, P., Xie, Z., Malovichko, M., Bhatnagar, A., Srivastava, S., & Conklin, D.J. Vascular Effects of Inhaled Crotonaldehyde in Mice: Role of TRPA1. *In revision*. #, co-first authors.
- Jin, L., Jagatheesan, G., **Lynch, J.**, Guo, L., & Conklin, D.J. (2020). Crotonaldehyde-induced Vascular Relaxation and Toxicity: Role of Endothelium and Transient Receptor Potential Ankyrin 1 (TRPA1). *Toxicology and Applied Pharmacology*. In press.
- **Finch, J.**, Riggs, D.W., O'Toole, T., Pope, C.A., 3rd, Bhatnagar, A., & Conklin, D.J. (2019). Acute exposure to air pollution is associated with novel changes in blood levels of endothelin-1 and circulating angiogenic cells in young, healthy adults. *AIMS Environmental Science*, 6(4): 265-276.
- Yeager, R., Riggs, D.W., DeJarnett, N., Tollerud, D.J., Wilson, J., Conklin, D.J., O'Toole, T., McCracken, J., Lorkiewicz, P., Xie, Z., Zafar, N., Krishnasamy, S., Srivastava, S., **Finch, J.**, Keith, R., DeFilippis, A., Rai, S., Liu, G., & Bhatnagar, A. (2018). Association Between Residential Greenness and Cardiovascular Disease Risk. *Journal of the American Heart Association*. 7(24): e009117.
- **Finch, J.** & Conklin, D. (2016). Air Pollution-Induced Vascular Dysfunction: Potential Role of Endothelin-1 (ET-1) System. *Cardiovascular Toxicology*, 16(3): 260-275.

Scientific Writing

- **Lynch, J.** & Conklin, D. (2020). Aldehydes & You: What You Should Know About Aldehydes and Cardiovascular Disease. *Sustain Magazine*, 42.

- **Lynch, J.**, Konkle, S., McGraw, K., Wheeler, J., Metcalf, H., & Heberle, L. (2020). What is the Status of the Lee's Lane Superfund Site? *Sustain Magazine*, 42.

Abstracts

American Heart Association Scientific Sessions

- Conklin, D.J., Jin, L., Whitehouse, K., Holbrook, D., **Finch, J.**, Lorkiewicz, P., Srivastava, S., Bhatnagar, A. (2019). Cardiorespiratory Responses to JUUL and Tobacco Cigarettes in Mice: Role of Acrolein.

American Public Health Association Annual Meeting

- DeJarnett, N., Yeager, R., Conklin, D.J., O'Toole, T., McCracken, J., Abplanalp, W., Riggs, D., Hamzeh, I., Wagner, S., Chugh, A., DeFilippis, A., Ciszewski, T., Wyatt, B., Becher, C., Hidgon, D., **Finch, J.**, Ismail, I., Tollerud, D.J., Rai, S., Shah, J., Zafar, N., Krishnasamy, S., Prabhu, S., Bhatnagar, A. (2015). Air Pollution and Tobacco Smoke Differentially Depress Circulating Angiogenic Cells in Humans.