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TOBACCO-DERIVED ALDEHYDES, PLATELETS, AND THROMBOGENICITY:  
ROLE OF TRANSIENT RECEPTOR POTENTIAL ANKYRIN-1

By

Andre D. Richardson

B.S., Nazareth College of Rochester, 2016

M.S., University of Louisville, 2020

A Dissertation

Submitted to the Faculty of the  
School of Medicine of the University of Louisville  
in Partial Fulfillment of the Requirements  
for the Degree of

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in Pharmacology and Toxicology

Department of Pharmacology and Toxicology  
University of Louisville  
Louisville, KY

August 2022



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## DEDICATION

This dissertation is dedicated to my entire family. Their encouragement, love, and support throughout my educational journey have helped to make this dream possible. I am the first in my family to pursue a doctoral degree, and I hope to make my family proud and to inspire our future generations to explore higher level education and careers.

## ACKNOWLEDGEMENTS

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If it were not for my fellow colleagues and peers, graduate school would be even more challenging. I would like to send a special thank you to my peers Dr. Christine Kim, Dr. Katlyn McGraw, Dr. Zimple Kurlawala, and Kyle Fulghum for always supporting me and for being present when I need a good laugh. I must also thank my friends and loved ones in the Louisville community. Thank you for providing me with an outlet beyond my studies to destress and enjoy my overall experience in Louisville, whether it be through sport or beyond.

Finally, my family has been my biggest support system. My mother is my sole inspiration for pursuing this career. Also, I strive to be a good example for my younger sister. I love you both! Thank you for giving me the courage and the resilience to further my education.

## ABSTRACT

### TOBACCO-DERIVED ALDEHYDES, PLATELETS, AND THROMBOGENICITY: ROLE OF TRANSIENT RECEPTOR POTENTIAL ANKYRIN-1

Andre D. Richardson

June 29, 2022

Cardiovascular disease (CVD) remains the world's leading cause of morbidity and mortality. Conventional smoking of tobacco cigarettes is the number one risk factor for onset of CVD. Active cigarette users as well as those individuals who are exposed to second-hand cigarette smoke are at increased risk of CVD. Studies have shown that conventional smoking significantly increases a user's risk of thrombosis, atherosclerosis, peripheral arterial disease, and coronary artery disease. Any of these diseases may trigger myocardial infarctions, stroke, or hypertension that may lead to cardiovascular morbidity and mortality.

New and emerging electronic nicotine delivery systems (ENDS), such as electronic cigarettes (e-cigs), are advertised as a less toxic alternative to smoking conventional, combustible tobacco cigarettes. To address potential toxicities associated with e-cig exposure, there are ongoing studies assessing CVD risk of e-cig use. This research includes studies of the chemical constituents present in the e-cig aerosols and their role in cardiovascular toxicity of exposure to e-cig-derived aerosols.

Epidemiological and experimental results suggest that harmful or potentially harmful constituents (HPHCs), such as aldehydes and flavorants, potentially mediate tobacco smoke- and e-cig aerosol-induced cardiovascular toxicity. Some HPHCs are shared among tobacco product types, including formaldehyde, acetaldehyde, acrolein, and menthol to name a few. However, their contribution to platelet activation, a marker of thrombosis, is largely unknown.

The transient receptor potential ankyrin 1 (TRPA1) channel is a sensory receptor that mediates some of tobacco smoke-induced cardiopulmonary toxicity, however, whether TRPA1 mediates platelet activation of tobacco smoke is unknown. Irritating aldehydes and flavorants such as acrolein, formaldehyde, and cinnamaldehyde are agonists of TRPA1. The goal of this dissertation research is to investigate the effects of e-cigs and their HPHCs on platelet activation and to test whether the TRPA1 channel mediates or contributes to tobacco-derived aerosols- or HPHC-induced platelet activation. Thus, the central hypothesis is that aldehydes (and flavorants) in tobacco-derived aerosols enhance platelet activation via stimulation of the TRPA1 channel.

## **Methods**

Healthy human volunteers as well as male and female mice were used for this research project. For the *ex vivo* studies, platelet-rich plasma (PRP) from healthy human volunteers was isolated and used in a platelet aggregometry assay. For this study, the effects of 15 different parent flavorants on adenosine diphosphate (ADP)-induced biphasic platelet aggregation were tested. For the *in vivo* studies, mice were exposed in whole-body inhalation chambers to mainstream cigarette smoke (MCS), e-cig aerosols, propylene glycol (PG), vegetable glycerin (VG), gaseous aldehydes, or HEPA-filtered air



for 4 days, 2 weeks, or 12 weeks. After euthanization, blood was drawn from the right ventricle, and platelet-leukocyte aggregates (PLAs) were measured by flow cytometry. In addition, plasma platelet factor 4 (PF-4) levels were measured by ELISA. Other endpoints in these studies include complete blood counts (CBCs), toxicology screening (Tox Screen), and vascular reactivity.

### ***Statistical analyses***

For the *ex vivo* studies involving the platelet aggregometry assay, the effects of each flavorant compound on ADP-induced human biphasic platelet aggregation were measured using a One-Way ANOVA or One-Way ANOVA with repeated measures where appropriate (SigmaPlot ver. 12).

For the *in vivo* studies using flow cytometry and PF-4 ELISA, the effects of whole-body exposure to individual HPHCs were compared with air exposures or across genotypes using the Mann-Whitney U test (SigmaPlot ver. 12) or the Two-Way ANOVA with Tukey post hoc test (GraphPad Prism ver. 9) where appropriate. Statistical significance was accepted where  $p < 0.05$ .

## **Results**

For the *ex vivo* studies in isolated human platelets, the direct effects of 15 different flavorant compounds (e.g., benzyl alcohol, eugenol, citronellol, menthol, menthone, diacetyl, maltol, limonene, methylbutyric acid, isoamyl acetate, acetylpyridine, eucalyptol, 2,5-dimethylpyrazine, cinnamaldehyde, and vanillin) on ADP-induced human platelet (biphasic) aggregation were investigated (**Chapter II**). PRP was incubated with 1 of 15 flavorants at 100  $\mu$ M for 5 min at 37 °C prior to the addition of

ADP (10  $\mu$ M). Aggregation curves were analyzed for flavorant-induced effects on total (%) aggregation, Phase 1 and Phase 2 components, and compared with their ADP-only control via One Way ANOVA with repeated measures. Our results show that eugenol significantly inhibited total aggregation in part by sole inhibition of the Phase 2 response. No other flavorant tested had significant effects.

For the *in vivo* studies, C57BL/6 male and female mice were acutely exposed to MCS, e-cig aerosols, PG:VG (30:70%, v:v) formaldehyde (FA; 2 or 5 ppm), acetaldehyde (AA; 5 ppm), acrolein (AC; 1 ppm), crotonaldehyde (CR; 1 or 3 ppm), or HEPA-filtered air (control) for 4 days, 2 weeks, or 12 weeks. Blood was isolated and PLAs were quantified as double positive events (CD41+/CD45+) via flow cytometry (**Chapter III**). These data reveal no statistically significant changes in % PLAs between exposures to e-cig aerosols or the saturated aldehydes (FA and AA) compared with filtered air control groups by the Mann-Whitney U test. There were, however, statistically significant changes in % PLAs in mice exposed to MCS, PG:VG, and the unsaturated aldehydes (AC and CR) when each was compared with its air control group via Mann-Whitney U test. In addition, results from PF-4 ELISA reveal that PG:VG exposure in female mice significantly elevated plasma PF-4 levels. Lastly, results show significant changes in % PLAs and plasma PF-4 levels between WT and TRPA1-null animals exposed to AC and CR (**Chapter IV**).

## **Summary & Conclusions**

Results of the *ex vivo* studies suggest that parent flavorant compounds commonly found in e-cig liquids neither activate nor inhibit ADP-induced human platelet

aggregation. As eugenol is a banned flavor, its inhibition of platelet activation proved a useful positive control. The results of *in vivo* studies support that short-term exposure of mice to unsaturated aldehydes but not the 2 most abundant saturated aldehydes lead to platelet activation, and thus, these aldehydes (AC and CR) may contribute to the overall MCS-induced thrombosis. Furthermore, the TRPA1 receptor appears to be an important mediator of unsaturated aldehyde-induced platelet activation *in vivo*.

Future studies should explore other agonists of platelet activation (as done with ADP) as well as explore the contribution of nicotine to better understand how HPHCs adversely impact thrombosis and overall cardiovascular health. More experiments are needed in order to understand the role of TRPA1 in the mechanism of tobacco smoke-induced thrombosis and CVD. For example, in isolated PRP, testing direct effects of HPHCs on platelet aggregation *in vitro* in the presence of a TRPA1 antagonist such as A967079 or HC030031. In conclusion, my research demonstrates that HPHCs (likely unsaturated aldehydes) are present in tobacco aerosols and at levels that activate platelets via stimulation of TRPA1. Thus, these HPHCs are toxic to the cardiovascular system and exposure to these aldehydes whether from e-cigs or combustible cigarettes increases CVD risk. These data regarding levels of unsaturated aldehydes that trigger platelet activation can help inform the FDA to better regulate tobacco products that emit HPHCs to lower levels that do not trigger pro-thrombotic changes.

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## CHAPTER I

### INTRODUCTION

#### **A. Background and Rationale**

##### **1. Cardiovascular disease & conventional tobacco smoking**

Cardiovascular diseases (CVD) are a wide range of illnesses impairing the strength and function of the heart and vasculature; it is the leading cause of death among people and in most racial ethnic groups in the United States [1] and worldwide [2]. Approximately 659,000 deaths (1 in every 4 deaths) are attributed to CVD in the United States per year [3]. CVD-related health expenditures and lost productivity are highest in the United States, totaling approximately \$316.1 billion in 2013 [4], \$363 billion in 2017 [3, 5], and overall cost is projected to be about \$818 billion by 2030 [6]. Thus, the health burden and the costs associated with CVD in the United States are significant. There is an urgent need to address causes and mechanisms of CVD in order to improve overall public health and reduce CVD-related burdens.

Onset of CVD is attributed to numerous risk factors including environmental exposures, age, sex, nutrition, dietary intake, alcohol consumption, physical inactivity, diabetic state or obesity, socioeconomic status, or pre-existing health conditions [7-11]. Conventional tobacco smoking is the most prominent modifiable risk factor of CVD [12-14]. More than 480,000 premature lives (approximately one-third of which are cardiovascular-related cases) are lost each year in the United States due to smoking [14,

15]. Conventional smoking alone causes atherosclerosis leading to cardiac arrhythmias [16], myocardial infarctions, and heart failure [17, 18]. Additionally, smoking increases atherosclerosis, i.e., deposits composed of fat and cholesterol in the vasculature, narrowing of the vasculature, and cause blockages in blood flow to and from the heart and throughout the body. Plaques act as a nidus for thrombotic events [14].

## **2. Platelet activation & conventional tobacco smoking**

When smoking causes blockages and impairs blood flow, this is in large part due to mainstream cigarette smoke (MCS)-induced platelet activation [19]. Tobacco smoke exacerbates plaque build-up in the vessels because of its chemical constituents (such as aldehydes or flavorants or nicotine) causing hypercoagulability due to platelet activation. First discovered by an Italian pathologist Giulio Bizzozero in 1882 [20], platelets are described as tiny (2-4  $\mu\text{m}$ ), anucleated, megakaryocyte-derived fragments circulating in the bloodstream [21]. These fragments have a special role in mediating inflammation, hemostasis, thrombosis, and blood coagulation as they serve to form blood clots or thrombi. Though synonyms of one another, there are distinct differences between a clot and a thrombus. A blood clot usually forms when there is vascular injury at a specific site [22, 23], from a cut for example. In a healthy blood vessel, though, a thrombus can form [24]. Factors that exacerbate risk of thrombus formation include irritant exposures such as cigarette smoke [25]. Furthermore, if part of a thrombus detaches and travels to distant extremities of the body via the bloodstream, this is called an embolus. Each of these solid masses are consequences of uncontrolled platelet activation. Uncontrolled platelet activation manifests in the form of deep vein thrombosis [26], pulmonary embolisms [26], myocardial infarctions [27], hypertension [28], or ischemic stroke [29]. Moreover,

cerebral stroke alone is characterized as an impairment of cerebral cognition and function due to lack of blood reaching the brain [30]. On average, a person dies of stroke every 3.5 minutes in the United States, which is approximately 411 stroke-related deaths per day [31]. It has also been reported that smokers have higher risks of stroke compared with never-smokers or former smokers [32-34].

Platelet activation is a predictive and prognostic biomarker of coronary artery disease, thrombosis, and subsequent CVD [35]. With specific attention to conventional cigarette smoke, as mentioned previously, research has shown that MCS induces coronary thrombosis [36, 37], a term used to describe thrombus formation. MCS activates platelets, increases platelet turnover, increases platelet volume in circulation, and increases spontaneous platelet aggregation [19, 38, 39]. By stimulating these events, MCS sensitizes platelets to a more hypercoagulable state in which clots can form throughout the human body to impair blood flow to vital organs and limbs. Thus, the severity of unwarranted platelet activation due to smoking puts a user at higher risk of poor cardiovascular (toxic) outcomes.

### **3. Comparison of conventional vs. electronic cigarettes**

More recently there has been a decline in use of conventional tobacco cigarettes [40], however, the use of new and emerging electronic nicotine delivery systems (ENDS), specifically electronic cigarettes (e-cigs), raises public health concerns. Since their initial marketing in the United States in 2007, the use of e-cigs has steadily increased. E-cig exposure has also been linked to increased risk of CVD [38]. Epidemiological studies report a rapid increase use of e-cigs with an initial claim that e-cigs were a healthier substitute in comparison to smoking conventional cigarettes [41-43]. As opposed to

conventional tobacco products, e-cigs do not typically contain tobacco unless it is used as a flavorant [44]. Advertisements in the form of social media and posters target youth and young adults to use e-cigs. In doing so, these products have gained substantial popularity and e-cig development and distribution companies have gained more wealth through its distribution to youth. With more use of these products, there is an increased potential for renormalizing use of conventional tobacco products overall [45]. This can furthermore increase CVD-risk.

There is a common misconception concerning e-cig use versus conventional cigarette use, specifically that exposure to e-cig aerosols is less toxic than exposure to MCS. Furthermore, the belief is that if e-cig aerosols are composed of far less non-carcinogenic chemicals than MCS, then e-cigs do not quantitatively produce highly toxic aerosols at the levels of conventional cigarettes [46]. Conventional tobacco cigarettes contain hundreds of ingredients (e.g. nicotine and cardiotoxic heavy metals), and during combustion, the smoke that is generated consists of a complex mixture of more than 7,000 toxic chemical constituents (e.g. aldehydes, polycyclic aromatic hydrocarbons, carbon monoxide, oxidants, ammonia, and tar) [47]. Approximately 70 of the known constituents of these products are known carcinogens and/or are poisonous [47]. On the other hand, e-cigs contain humectants (or e-liquids) with the following ingredients: propylene glycol (PG), vegetable glycerin (VG), nicotine, and various flavorants [48]. Around the time e-cigs were introduced, these e-liquids were thought to be nontoxic because either they did not contain toxic by-products, or they did not produce these toxins at levels near those present in or derived from tobacco combustion [49, 50]. However, research has shown that there are shared harmful or potentially harmful

constituents (HPHCs) such as aldehydes and flavorants present in aerosols of both types of tobacco products [51]. Additionally, nicotine is an addictive substance present in both aerosols, and it also has known health risks [52-57].

Recent studies have provided evidence that e-cig aerosol or extract induces thrombosis. Qasim *et al.* finds that acute exposure of mice to e-cig aerosol shortens tail bleeding time caused by platelets clotting faster, augments platelet degranulation and aggregation, increases expression of surface markers, and enhances phosphorylation of Akt and ERK that support thrombus formation and stability [58]. Popular e-cig brands like JUUL also enhance platelet reactivity. Montes Ramirez *et al.* find that exposure of mice to JUUL-derived aerosols (70 puffs/d; 2wk) also shortens tail bleeding time, augments platelet degranulation and secretion, enhances adenosine diphosphate- and thrombin-induced platelet aggregation, and enhances phosphatidylserine expression on platelets [59]. Hom *et al.* find that isolated platelets from healthy human volunteers (n=50) exposed to e-cig extracts also enhance platelet activation, adhesion, and aggregation *ex vivo* [60]. This study involved exposing human platelets to extracts of conventional cigarettes and e-cig aerosols and find that both exposures induced platelet activation without nicotine [60]. Collectively, these studies provide evidence that e-cigs may enhance thrombotic outcomes in murine and human models.

It is unclear whether platelet activation is of equal concern with conventional cigarette use as with e-cig use in humans. Nocella *et al.* collected blood from 40 self-reported healthy participants that are composed of age-matched smokers and non-smokers [61]. As an acute study, these participants gave blood 5 mins before and after smoking or vaping. The expression of sCD40L and sP-selectin (platelet biomarkers) on

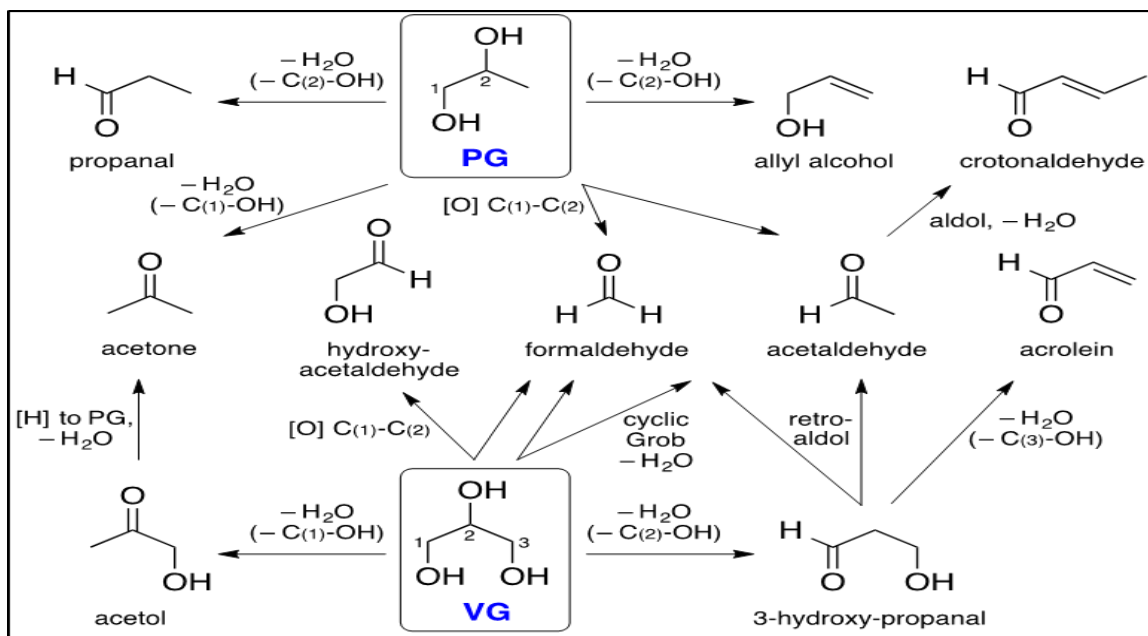
the surface of activated platelets was measured via ELISA. Furthermore, isolated platelets were analyzed for collagen (2 µg/mL)-induced platelet aggregation *ex vivo* via an aggregometry assay. There were no statistical differences in abundance of surface markers on platelets between e-cig and conventional cigarette users after vaping or smoking, but Nocella *et al.* finds that platelet aggregation is significantly higher after conventional cigarette use compared with e-cig use both in non-smokers and smokers [61]. Thus, the authors conclude that platelet reactivity is higher with use of conventional cigarettes than use of e-cigs [61]. In another study, Metzen *et al.* collected blood samples from 212 self-report healthy participants that are composed of non-smokers, e-cig users, and conventional cigarette users. The authors performed Multiplate platelet aggregometry assays in isolated platelets via adding collagen and adenosine diphosphate agonists to compare levels of platelet sensitivity between each group [62]. Their results indicate platelet aggregation in vapers is significantly higher than in non-smokers as well as being higher than in conventional cigarette users [62]. The authors conclude that platelet reactivity to collagen and adenosine diphosphate is higher in e-cig users than conventional cigarette user. [62]. The results of these two publications suggest there is a critical need to assess thrombogenic risk associated with using new and emerging tobacco products. Similarly, knowledge of the chemicals (constituents) in these aerosols that contribute to platelet activation, and at what level, will be useful because the FDA can regulate these products to reduce harm. Moreover, the specific mechanisms by which these constituents lead to platelet activation could help in developing interventions that may reduce the risk of stroke/thrombus formation in users who continue to vape and/or smoke tobacco products.

#### 4. Harmful or Potentially Harmful Constituents (HPHCs): aldehydes & flavorants

Conventional tobacco cigarettes are combustible products that generate an aerosol containing many HPHCs including reactive aldehydes [63]: formaldehyde, acetaldehyde, acrolein, and crotonaldehyde. E-cigs are non-combustible products. When taking a puff from an e-cig, the e-liquid components within the cartridge are heated and generate a vapor containing the same toxic reactive aldehydes as a result of a thermal dehydration reaction of the e-liquids [64]. Although acrolein and crotonaldehyde are not typically produced in e-cig aerosols at high levels, there are proposed mechanisms by which PG:VG produces these unsaturated aldehydes as well (**Figure 1**) [65]. Thermal degradation of VG produces acrolein, the simplest  $\alpha,\beta$ -unsaturated aldehyde [65, 66], while degrading PG generates saturated aldehydes: formaldehyde, acetaldehyde, and propionaldehyde [65, 66]. Varying concentrations of these aldehydes, in aerosol, are generated depending on the e-cig battery voltage [64, 67, 68], ratio of PG:VG [69], and use topography [70, 71]. Furthermore, the various concentrations of aldehydes in e-cig aerosols are associated with a number of CVD risk factors such as increased platelet counts and increased blood coagulation via platelet activation [72]. Alarabi *et al.* discusses the role of ENDS products in potentiating thrombosis [73]. According to the recent data they have reviewed, e-cig use can induce inflammation, oxidative stress, and thrombosis in a similar fashion as does use of conventional products, adding that each toxicity potentiates one another in a cyclical manner [73]. Additionally, even different PG:VG formulations (i.e. 30:70% PG:VG, or 50:50% PG:VG ratios) have thrombotic consequences including increased granule secretion, increased CD40 and P-selectin



expression, and decreased clotting time [74]. As mentioned previously, each of these is a marker of platelet activation from exposure to PG:VG-derived aerosols.



**Figure 1.** Theoretical thermal- and/or heating coil-induced generation of carbonyl compounds from propylene glycol (PG) and vegetable glycerin (VG) [65].

Although many health implications have been discussed concerning aldehydes derived from e-cig aerosols, there is not a defined relationship between the toxic aldehydes and the risk these impose on CVD. Acrolein (AC), an  $\alpha,\beta$ -unsaturated aldehyde, is a common air pollutant that has long-been associated with increased CVD risk [75, 76] and has been shown to increase thrombosis and activate hemostasis via platelet activation [77], promote platelet-leukocyte aggregate formation, and also form adducts with platelets [77], though the mechanism is unclear.

Crotonaldehyde (CR) is an  $\alpha,\beta$ -unsaturated aldehyde with a pungent, suffocating odor. The Occupational Safety and Health Administration (OHSa) imposed a 2 ppm exposure limit to CR over an eight-hour work shift, but levels between 0.035 ppm and 0.12 ppm induce toxicity [78]. Inhaled CR is toxic at low concentrations and causes irritation within the upper respiratory tract [79]. At the cardiovascular level, our research shows that CR decreases blood pressure and induces endothelium- and nitric oxide (NO)-dependent relaxation in the vasculature [80, 81]. Although much remains unknown about the cardiotoxicity associated with CR exposures, we hypothesized that this compound could potentially induce similar effects as acrolein due to similarities in their chemical structures.

Formaldehyde (FA) is the simplest saturated aldehyde and is gaseous at room temperature; it is colorless with a pungent, irritating odor. According to the National Toxicology Program (NTP), FA is a known human carcinogen [82]. In terms of the cardiotoxic effects that are associated with FA exposure, it has been shown that high levels of FA induce vasopressor effects in anesthetized rats, or an increase in blood pressure as a result of a blood vessel undergoing contraction [83]. Conversely, lower

levels of FA in rats result in hypotension [83]. Acetaldehyde (AA), a saturated aldehyde, is a reasonably anticipated human carcinogen [84]. It has also been shown that AA induces hypertension at high concentrations ( $\geq 3$  ppm) in inhalation-exposed rats [85]. Similar results are observed when AA is administered via intraperitoneal injection (5-20 mg/kg) with a decrease in heart rate [86, 87]. In our studies, our investigations focused on AC, CR, FA, and AA because these toxic aldehydes are present in tobacco and e-cig aerosols, and thus, may contribute to enhance CVD-risk [88].

Flavors also contribute to youth use of e-cig products and renormalization of conventional tobacco use [89]. A variety of flavors, like bubble gum or cherry, attract youth to try e-cigs to a sweet flavored e-cig for consumption. What is believed as a “bubble gum” flavor is often a flavor profile composed of numerous chemicals. Flavor profiles (>7,700) are a product of mixing a variety of chemical compound additives (flavorants) including pure chemicals such as menthol, benzaldehyde, eugenol, cinnamaldehyde, or ethylvanillin [90]. When heated using an e-cig, the chemical products (e.g. flavor acetals) may exacerbate CVD risk [66, 91]. This alone creates new alarms in that flavorings may contribute to adverse cardiovascular outcomes. Fetterman *et al.* exposed human aortic endothelial cells to nine different flavoring compounds and found that these flavors induced dysfunction to the endothelium via cell death, oxidative stress, increased inflammation, and decreased nitric oxide (NO) production [92]. To date, no evidence had been shown regarding effects of e-cig-derived flavorants on platelet activation.

## **5. Transient Receptor Potential Ankyrin-1**

Although we know CVD is a serious adverse outcome of tobacco smoking, the mechanisms by which these aerosols and their constituents induce CVD are largely unknown. Acrolein, an unsaturated aldehyde and constituent of cigarette smoke, is a known cardiopulmonary toxicant that activates the transient receptor potential ankyrin 1 (TRPA1) channel. TRPA1 is a non-selective, permeable  $\text{Ca}^{2+}$  channel and a promiscuous sensory receptor located in sensory fibers, vasculature, vascular smooth muscle cells, endothelium, urothelium, and in the heart [80, 93-99]. There is no documentation of TRPA1 expression on platelets, specifically. However, activation of this channel in other tissues (lung, blood vessels, sensory fibers) may elicit effects on platelets by an indirect mechanism.

Along with its activation by acrolein, TRPA1 is also activated by noxious irritants, environmental toxicants, diesel exhaust, flavor compounds like menthol from mint, cinnamaldehyde from cinnamon, and allicin from garlic, allyl isothiocyanate (an ingredient in wasabi and mustard), and cigarette smoke and its constituents [100-103]. TRPA1 on sensory nerve fibers mediates pain-reception/transmission, and its activation induces pulmonary reflexes (i.e. sneezing, coughing, respiratory breaking), production of reactive oxygen species (ROS), body temperature regulation, blood pressure regulation, and heart rate regulation [95, 104-106]. The mechanism of triggering TRPA1 activation includes opening of the channel that allows calcium entry into a cell. When TRPA1 is activated and calcium enters a cell, there are several pathways by which calcium induces cell-specific activity. In endothelial cells, calcium entry activates endothelial nitric oxide synthase (eNOS) to make nitric oxide (NO). When calcium enters a sensory neuron, it

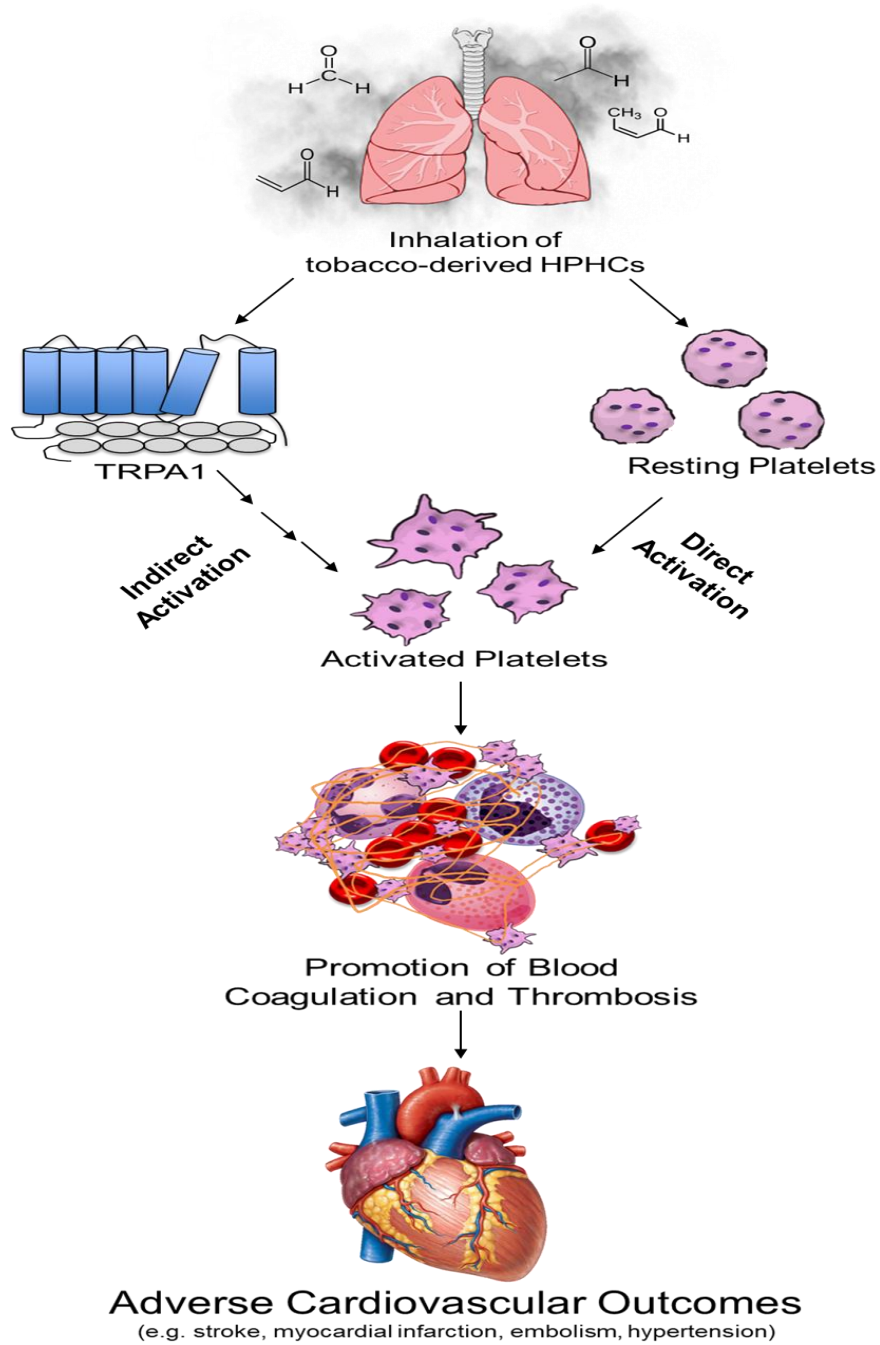
can simultaneously cause depolarization and a neuronal generator potential. This ultimately leads to action potential and pain signaling/sensation/perception and causes release of calcium-dependent vesicles that typically contain calcitonin gene-related peptide (CGRP) and substance P (subP) [107, 108]. These peptides have been implicated in pro-inflammatory effects of TRPA1 agonists such as increased blood flow, increased vascular permeability, and attraction of leukocytes to area of injury [109]. Because platelet activation is also a pro-inflammatory effect, TRPA1 may be involved with platelet actions. Additionally, acrolein induces adverse TRPA1-dependent cardiovascular outcomes [110-113], though the role of TRPA1 in mediating other aldehyde-induced thrombosis is largely unknown. This dissertation investigates the role of TRPA1 in mediating HPHC-induced platelet activation and thrombosis as an outcome observed with conventional cigarette and e-cig use.

In summary, CVD is a worldwide dilemma that is, in large part, due to use of tobacco products. Exposures to conventional tobacco smoke can increase CVD-risk and perhaps this is also true of e-cig aerosols. There is also evidence that these exposures can exacerbate platelet activation leading to unwarranted blood coagulation in the vasculature, although the specific mechanisms are unclear. Tobacco-derived aerosols contain HPHCs that historically affect the cardiovascular system. This dissertation serves to elucidate the effects of aldehyde and flavorant constituents in tobacco-derived aerosols on platelet activation and thrombosis, in part by interrogating whether TRPA1 is a potential mechanism for inducing observed effects. Understanding the contribution of individual HPHCs to CVD allows for the U.S. Food and Drug Administration to better regulate levels of these compounds present and generated in and by tobacco products.

Understanding the role of TRPA1 allows us to better understand the mechanism by which these compounds exert their toxicity, and this understanding may aid in development of pharmaceutical interventions to minimize CVD-related health burdens.

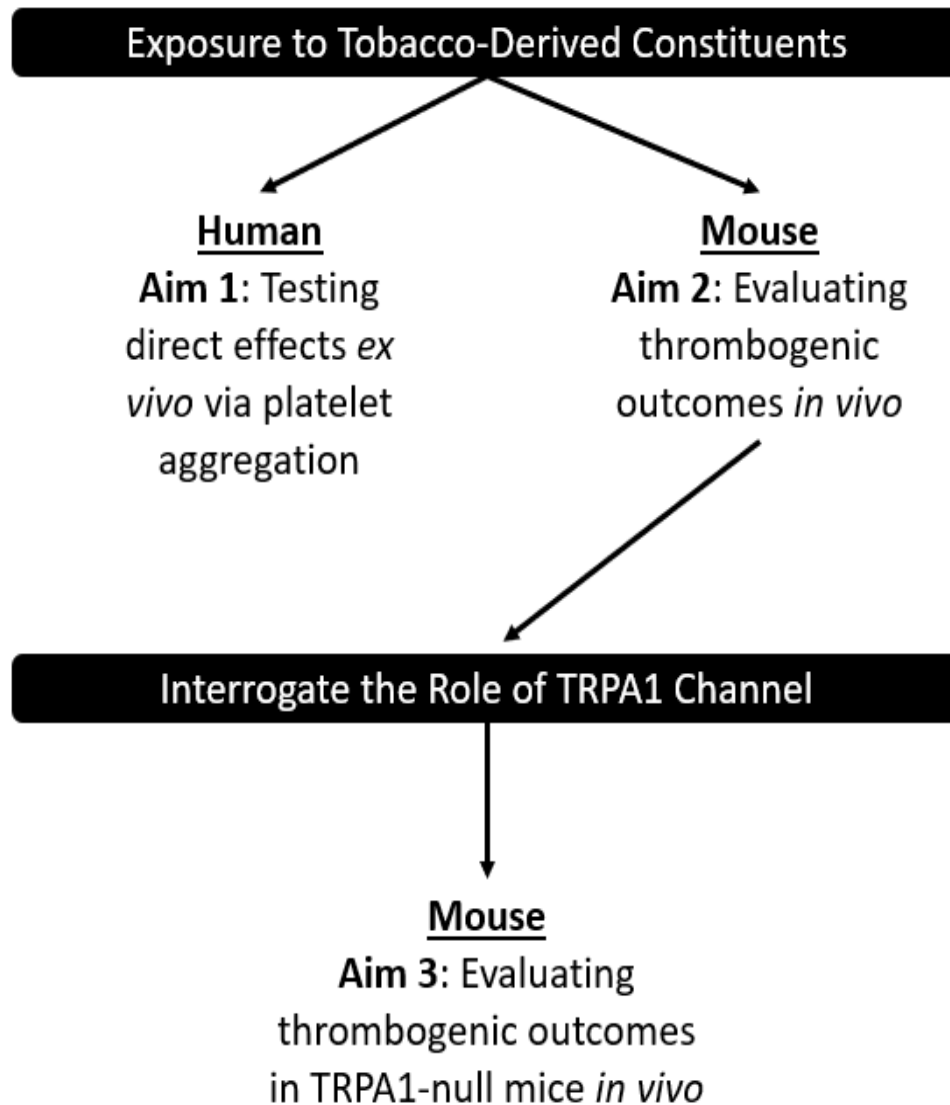
## **6. Statement of Goals**

The primary goal of this dissertation research is to determine how tobacco-derived aldehydes and flavorants affect platelet activation. Based on review of previous literature, we know that each compound can induce a cardiovascular toxicity, but minimal knowledge with regards to platelet pathophysiology. Additionally, we do not fully understand mechanisms that contribute to tobacco-induced CVD, though we know researchers have hypothesized that effects related to exposure to some aldehydes such as acrolein are mediated via TRPA1-dependent activation. Thus, our central hypothesis is that tobacco and e-cig-derived aldehydes and flavorants are platelet activators and alter platelet biology via the TRPA1 pathway, directly or indirectly (**Figure 2**). To test this hypothesis, we explored the following aims (**Figure 3**):



**Figure 2.** Hypothesis of tobacco-derived HPHCs (aldehydes or flavorants)-induced platelet activation and subsequent thrombosis.





**Figure 3.** Evaluating the effects tobacco-derived constituents on platelet activation and pro-thrombotic outcomes.

## **B. Specific Aims**

### **1. Aim 1. Determine the direct effects of individual HPHCs on platelet aggregation *ex vivo*.**

Platelets are the key mediators in hemostasis and thrombosis. When platelets become activated, they undergo aggregation in which these fragments bind to each other. This is vital for formation of the thrombus or platelet plug in order to cease bleeding. The function of platelets has long been studied via a number of different assays and methodologies. The platelet aggregometry assay is the most widely accepted, gold standard, platelet function assay that utilizes human platelet rich plasma (PRP) isolated from whole blood. This method measures the ability of different agonists to induce platelet-platelet aggregation *in vitro*. For our studies, we used a platelet aggregometry assay to assess the effects of HPHCs (specifically flavorants) on adenosine diphosphate (ADP)-induced human platelet aggregation *ex vivo*. We hypothesized that direct exposure of flavorants promotes platelet aggregation in our model.

### **2. Aim 2. Quantify the level- and time-dependence of aldehyde exposure on prothrombotic alterations *in vivo*.**

We hypothesized that exposure to tobacco-derived aerosols enhanced platelet function and activation. In this study, C57BL/6 male and female mice underwent whole body exposures to HEPA-filtered air (control; 6 h/d) MCS (12 cigs/d), e-cig aerosols (6 h/d), AC (1 ppm), CR (1 & 3 ppm), FA (2 & 5ppm), or AA (5ppm) for 4 days (or CR for 12 weeks) and look quantitatively for changes in percentage of platelet-leukocyte aggregate (PLA) formation, a marker of thrombosis, via flow cytometry analyses. We also

observed the total clotting time from the mouse tail vein post-exposure using tail bleeding time assays.

### **3. Aim 3. Test the role of the TRPA1 channel.**

We hypothesized that the thrombotic outcomes associated with aldehyde exposures may occur via the TRPA1 pathway. Using TRPA1-null mice *in vivo* under conditions that showed significant changes in % PLAs and clotting times to see if those outcomes are TRPA1-dependent. Thus, we will test if the HPHCs enhance platelet activation via a TRPA1-mediated mechanism.

## CHAPTER II

### EFFECT OF ELECTRONIC CIGARETTE FLAVORANTS ON HUMAN PLATELET AGGREGATION *EX VIVO*

#### INTRODUCTION

Electronic cigarettes (e-cigs) or electronic nicotine delivery systems (ENDS) are marketed as “healthier alternatives” to conventional tobacco cigarettes, yet the cardiovascular disease (CVD) risk associated with chronic e-cig use is unknown. Moreover, given that e-cigs use has increased dramatically among youth and young adults, there are concerns about renormalization of tobacco use and the potential increased CVD risks associated with acute and chronic use of e-cigs. From 2017 to 2018, the use of e-cigs among high school and middle school students increased from 11.7% to 20.8% and 3.3% to 4.9%, respectively [114]. In 2021, the National Youth Tobacco Survey in the United States reported that e-cigs are the most commonly used form of tobacco products among youth, with an estimated 2.06 million students documented as current e-cig users [115]. Additionally, 84.7% of those students use flavored e-cigs, primarily fruit-flavored, and thus, the increase in youth use of e-cigs appears to be due, in large part, to the many flavors offered, including e-cigs with appealing names such as “Cotton Candy,” “Gummi Bear,” and “Bubble Gum” [89, 115]. Flavor profiles (>7,700) are a product of mixing a variety of chemical compound additives (flavorants) including pure chemicals such as menthol, benzaldehyde, eugenol, cinnamaldehyde, or ethylvanillin [116]. These compounds may be at relatively high concentrations (0.1-1% v/v) with cinnamaldehyde being present at up to 4% [89, 117, 118]. Although many of these flavoring compounds are generally regarded as safe (GRAS) for ingestion, there is little known about potential toxicity of heated

flavorants as inhaled during typical e-cig use. Notably, commonly used e-cig-derived flavoring compounds, without nicotine, induce concentration-dependent increases in reactive oxygen species (ROS) and cytotoxic effects in human monocytic cells [119]. Specifically, Muthumalage *et al.* (2018) report that cinnamaldehyde and vanillin are more toxic than other flavorants tested because cinnamaldehyde and vanillin exceed the level of toxicity, e.g., cell viability, of other flavorants (e.g., diacetyl, acetoin, maltol, pentanedione, *o*-vanillin, and coumarin) in human monocytic cells for induction of oxidative and inflammatory responses [119].

It is critical to assess overall CVD risk of e-cig use, and thus, there is a need to assess the contribution of individual constituents in e-cigs such as flavorants to the overall CVD risk [120]. For example, exposure of human endothelial cells to each of nine different flavoring compounds led to concentration-dependent reduction in nitric oxide (NO) production and concurrent increases in markers of oxidative stress, inflammation, and apoptosis [92]. As NO inhibits platelet activation [121], flavorants that diminish endothelial production of NO may increase the risk of platelet activation [122]. In fact, acute exposure of mice to e-cig aerosols induces prothrombotic effects [58]. Inhalation exposures *in vivo* are complex, though, and thus, use of a more direct, higher throughput *in vitro* assay may allow for identification of individual e-cig flavorants that exert platelet toxicity.

Despite this urgent need to evaluate e-cigs and their constituents for prothrombotic effects, there has been little investigation into whether flavorants directly induce platelet activation. The present study investigated how unaltered parent flavorants that are commonly found in e-cig liquids may influence prothrombotic events, specifically

platelet aggregation. We hypothesized that direct exposure of platelets to the parent flavorings induces significant changes in biphasic platelet aggregation *ex vivo*. Thus, the goal of this study was to quantify the effects of common e-cig flavorants on adenosine diphosphate (ADP)-induced human platelet aggregation. Furthermore, we investigated whether flavorants would affect either platelet initiation (phase 1) or secondary aggregation and amplification (phase 2) to better understand direct actions of flavorants.

## MATERIALS AND METHODS

### *Materials*

Flavorants: The following 15 e-cig flavorants were used in this study (**Figure 4**): benzyl alcohol, citronellol, eugenol, menthol, cinnamaldehyde, vanillin, isoamyl acetate, eucalyptol, limonene, diacetyl, menthone, methylbutyric acid, 2,5-demethylpyrazine, acetylpyridine, and maltol. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. ADP was purchased from Chrono-log Corp. (Havertown, PA). A 1 mM stock solution was made by reconstituting 2.5 mg of lyophilized ADP in sterile saline and stored at -80 °C until used.

Preparation of Flavorants: Each parent flavorant was prepared fresh before testing and dissolved in ethanol to obtain stock solution. Ethanol was used as vehicle control.

Humans: Blood (~15 mL per donor) was collected into tubes containing 3.8 % trisodium citrate from self-reported healthy male and female human volunteers at the University of Louisville Health Sciences Center campus. This exempt protocol was approved by the University of Louisville Institutional Research Board, and no participant information was disclosed to investigators.

### *Methods*

Preparation of Human Platelets: Samples were analyzed within 3h of blood draw. Samples were gently mixed for 5 min on a tube rocker at RT. To obtain PRP, citrated blood samples were centrifuged (180xg; 15 min; RT). Remaining blood was centrifuged

again (1,300xg; 20 min; RT) to obtain platelet-poor plasma (PPP). PRP was adjusted to  $3 \times 10^8$  PLT/mL with PPP used as the reference sample.

*Platelet Aggregometry Assay:* The Born method of turbidimetric aggregation was used [123]. A four-channel Chrono-log Lumi-aggregometer 480VS was used to measure aggregation photometrically over 5 min post ADP-induction (10  $\mu$ M; **Figure 5**). PRP or PPP reference samples (350  $\mu$ L) were incubated at 37 °C in cuvettes (Chrono-log, P/N 312) with a stir bar (Chrono-log, P/N 311; 1,000 rpm). Flavorants at 100  $\mu$ M (or eugenol at 10-100  $\mu$ M) or ethanol control were added in PRP and pre-incubated (37 °C) for 5 min. After pre-incubation, ADP was added to stimulate biphasic aggregation over 5 min. ADP initiated a rapid shape change followed by onset of rapid aggregation (phase 1, P1) and then greater aggregation and amplification (phase 2, P2) responses.

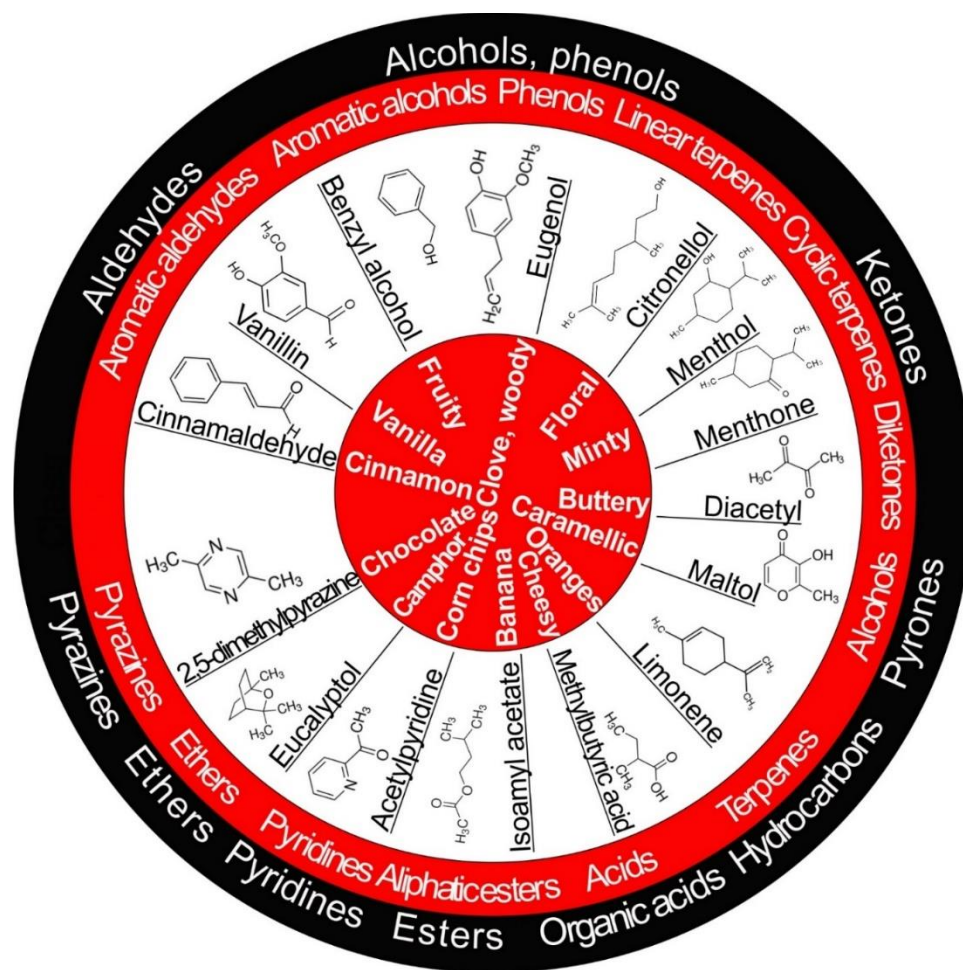
*Calculations:* ‘Total aggregation’ was defined as the final aggregation (%) at 5 min after ADP (**Figure 5**). P1 (%) was defined from onset of aggregation until an inflection point or “leveling off” of the initial P1 slope. P2 (%) was calculated as the difference between Total aggregation and P1 (Total aggregation - P1 = P2). For example, if a compound had anti-aggregation activity, then the P2 value could be negative. To account for variation in participants’ baseline aggregation responses to ADP, both P1 and P2 values also were normalized as a percentage of the total aggregation to 100 %.

### ***Statistical analyses***

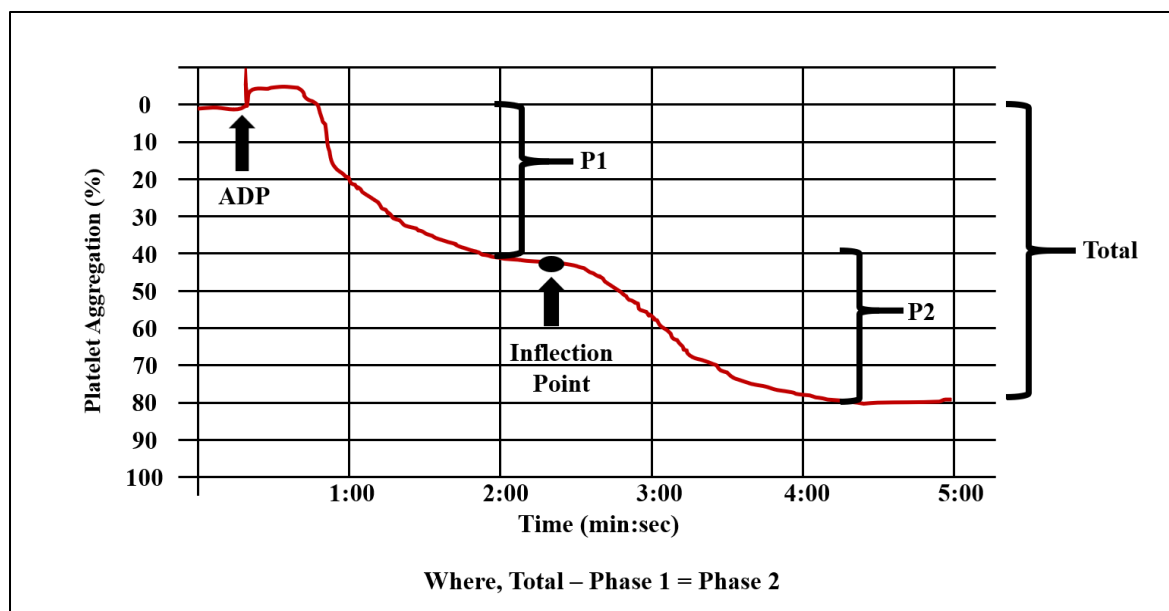
Total, P1, and P2 aggregation values are means  $\pm$  SE. Percentage responses for Total aggregation, P1, and P2 were compared between control (ADP only) and each flavorant



compound using a One-Way ANOVA (or One-Way ANOVA with repeated measures for eugenol) (SigmaPlot ver. 12). Statistical significance was accepted at  $p < 0.05$ .



**Figure 4.** Electronic cigarette flavorant additives used in this investigation, organized by flavor profile (innermost circle), compound nomenclature, compound structure, structural group, and chemical class (outermost circle) [124].



**Figure 5.** Schematic of adenosine diphosphate (ADP)-induced phase 1 and phase 2 human platelet aggregation ex vivo. A flavorant is added to a sample of PRP and is then placed into the aggregometer for 5 minutes of incubation at 37°C. Addition of ADP stimulates rapid change of platelets from a quiescent state to an activate state, causing phase 1 aggregation in which platelet alpha and dense granules to secrete. This initial release of granules subsides (inflection point), and the secreted granules activate neighboring platelets, thus amplifying a secondary aggregation response (phase 2). These aggregating responses occur within 5 minutes before stabilizing. Total aggregation (%) is recorded after 5 minutes. The inflection point is recorded as the sole response of phase 1 (%). Phase 2 (%) response is calculated as Total minus Phase 1 [124].

## RESULTS

### *Direct effect of flavoring additives on ADP-induced platelet aggregation in human PRP*

We tested 15 flavorants at 100  $\mu$ M for their direct effects on platelet aggregation induced by ADP (**Table 1**). Except for eugenol, none of the other 14 flavorants had any effect on total percentage of ADP-induced aggregation [124]. Eugenol, in contrast to all other flavorants tested, had a strong inhibitory effect on total platelet aggregation (**Table 2**) [124].

### *Total aggregation as function of Phase 1 and Phase 2 responses*

We recorded the responses of platelets due to ADP stimulation as a collective in real-time. The total aggregation through 5 min has 3 distinct elements: 1) initial shape change (addition of agonist); 2) initiation of platelet activation (P1); and 3) feed forward aggregation due to platelet secretion of granule contents including ADP, serotonin, and thromboxane that further stimulate aggregation (P2). In quantifying these phases, ADP alone stimulated  $57 \pm 2\%$  of total aggregation in P1 (**Table 1**). The remaining fraction was P2 ( $43 \pm 2\%$ ). Because most flavorants tested had no effect on total aggregation, we did not expect, nor did we find, any effect of most flavorants on either P1 or P2 % aggregation (**Table 1**).

In contrast, as eugenol significantly decreased total aggregation, we observed that all of this inhibitory effect resulted from inhibition of P2 response (**Table 2**). The inhibitory effect on P2 was concentration-dependent and was significant even at the lowest concentration tested of 10  $\mu$ M. ADP alone control stimulated  $62 \pm 3\%$  of total aggregation in P1 (**Table 2**). The remaining fraction was P2 ( $38 \pm 3\%$ ). In the presence

of eugenol, ADP stimulated a normal P1 aggregation yet P2 was blunted in a concentration-dependent manner. These results indicated that P2 was inhibited by eugenol. At the highest eugenol concentration tested (100  $\mu$ M), we observed disaggregation in which our traces reversed post-P1 activity resulting in negative P2 values (**Table 2**). Together, these data indicate eugenol is an inhibitor of ADP-induced platelet aggregation.

**Table 1.** Effect of flavorants (100  $\mu$ M) on ADP-induced (10  $\mu$ M) human platelet total and biphasic aggregation *ex vivo*.

Flavorant	N	100 $\mu$ M			Normalized to 100% aggregation	
		Total (%)	Phase 1 (%)	Phase 2 (%)	Phase 1 (%)	Phase 2 (%)
Control	24	65 $\pm$ 3	37 $\pm$ 2	28 $\pm$ 2	57 $\pm$ 2	43 $\pm$ 2
Cinnamaldehyde	16	63 $\pm$ 3	38 $\pm$ 2	25 $\pm$ 3	61 $\pm$ 3	39 $\pm$ 3
Menthol	21	61 $\pm$ 6	36 $\pm$ 2	26 $\pm$ 2	58 $\pm$ 2	42 $\pm$ 2
Benzyl Alcohol	11	64 $\pm$ 3	38 $\pm$ 2	27 $\pm$ 2	59 $\pm$ 2	41 $\pm$ 2
Vanillin	21	61 $\pm$ 2	35 $\pm$ 1	26 $\pm$ 2	58 $\pm$ 2	42 $\pm$ 2
Maltol	6	61 $\pm$ 4	34 $\pm$ 1	27 $\pm$ 3	56 $\pm$ 2	44 $\pm$ 2
Limonene	7	61 $\pm$ 3	37 $\pm$ 2	24 $\pm$ 1	61 $\pm$ 2	39 $\pm$ 2
Menthone	13	65 $\pm$ 3	35 $\pm$ 2	30 $\pm$ 2	54 $\pm$ 2	46 $\pm$ 2
Methylbutyric acid	14	67 $\pm$ 3	36 $\pm$ 2	31 $\pm$ 2	54 $\pm$ 2	46 $\pm$ 2
Diacetyl	16	63 $\pm$ 2	37 $\pm$ 2	26 $\pm$ 2	59 $\pm$ 3	41 $\pm$ 3
Acetylpyridine	14	61 $\pm$ 3	33 $\pm$ 1	28 $\pm$ 3	56 $\pm$ 2	44 $\pm$ 2
Citronellol	8	63 $\pm$ 3	38 $\pm$ 3	25 $\pm$ 2	61 $\pm$ 3	39 $\pm$ 3
Eucalyptol	18	62 $\pm$ 2	33 $\pm$ 1	29 $\pm$ 2	54 $\pm$ 2	46 $\pm$ 2
Isoamyl acetate	14	60 $\pm$ 3	34 $\pm$ 2	26 $\pm$ 2	57 $\pm$ 2	43 $\pm$ 2
2,5-dimethylpyrazine	12	63 $\pm$ 2	35 $\pm$ 1	29 $\pm$ 2	55 $\pm$ 2	45 $\pm$ 2

Data are mean  $\pm$  SEM. Abbreviations: ADP, adenosine diphosphate; N, number of human subjects. Total aggregation (%) - Phase 1 (%) = Phase 2 (%) [124].

**Table 2.** Concentration-dependent effects of eugenol on ADP-induced (10  $\mu$ M) human platelet total and biphasic aggregation *ex vivo*.

Eugenol [ $\mu$ M]	N	Eugenol+ADP			Normalized to 100% aggregation	
		Total (%)	Phase 1 (%)	Phase 2 (%)	Phase 1 (%)	Phase 2 (%)
<b>0; Control</b>	24	66 $\pm$ 4	41 $\pm$ 3	25 $\pm$ 3	62 $\pm$ 3	38 $\pm$ 3
<b>10</b>	9	49 $\pm$ 2*	38 $\pm$ 3	13 $\pm$ 4*	79 $\pm$ 4*	21 $\pm$ 4*
<b>25</b>	12	42 $\pm$ 3*	43 $\pm$ 2	-1 $\pm$ 1*	99 $\pm$ 3*	1 $\pm$ 3*
<b>100</b>	17	41 $\pm$ 2*	43 $\pm$ 2	-2 $\pm$ 1*	106 $\pm$ 3*	-6 $\pm$ 3*

Data are mean  $\pm$  SEM. Abbreviations: ADP, adenosine diphosphate; N, number of human subjects. Total aggregation = Phase 1 (%) + Phase 2 (%). \*P<0.05, One-Way ANOVA with repeated measures [124].

**Table 3.** Levels of common flavorants in electronic cigarette liquids, and the estimated blood levels of flavorants of e-cigarette users.

<b>Flavorant (M.W.)</b>	<b>Flavorant [mg/mL]</b>	<b>~µg per puff<sup>a</sup></b>	<b>~blood [µg/dL]<sup>b</sup></b>	<b>~blood [µM]<sup>c</sup></b>	<b>Ref.</b>
Cinnamaldehyde (132.16)	0.8 – 155	310	310	23.46	[89, 125]
Menthol (156.27)	0.1 – 84	168	168	10.75	[89, 117, 126]
Benzyl Alcohol (108.14)	0.1 – 39	78	78	7.21	[89, 125]
Vanillin (152.15)	0.1 – 33	66	66	4.33	[89, 126]
Eugenol (164.20)	1.9 – 12	24	24	1.46	[89, 125]
Maltol (126.11)	0.8 – 4.9	9.8	9.8	0.77	[89, 125]
Limonene (136.24)	0.7 – 2.9	5.8	5.8	0.43	[89]
Menthone (154.25)	0.6 – 1.4	2.8	2.8	0.18	[89, 125]
Methylbutyric acid (102.13)	0.6	1.2	1.2	0.12	[89]
Diacetyl (86.09)	0.0004 – 0.24	0.48	0.48	0.006	[126]

<sup>a</sup> Estimated µg of flavorant aerosolized in a puff. Estimated µg was based on volume of e-liquid consumed per puff \* [flavorant]. <sup>b</sup> For simplicity, we assumed 100 % of aerosolized flavorant was absorbed into 100 ml blood. <sup>c</sup> To calculate flavorant [molar], blood concentration was divided by flavorant M.W [124].



## DISCUSSION

### *Purpose of Study*

To our knowledge, no one has investigated the direct effects of these 15 common flavorants on ADP-induced human platelet aggregation. We hypothesized that direct exposure of platelets to flavorants may enhance ADP-induced platelet aggregation *ex vivo* by altering either or both of the aggregation phases. Under the conditions used, our data indicate that the flavoring compounds tested do not influence direct platelet aggregation induced by ADP. Importantly, eugenol, a known anticoagulant and a banned tobacco flavorant compound, acts as a strong positive control. Our results demonstrate that the conditions used herein are appropriate to detect either activation or inhibition of ADP-induced platelet aggregation by common e-cig flavorants.

### *Strengths of Study*

This study has several strengths: 1) human platelets; 2) wide range of common flavorants tested; 3) appropriate test concentration; and, 4) a strong positive control flavorant.

*Human Platelets:* Both animal and *in vitro* research models are on the rise to reduce the need of human-based studies. However, research using human samples is most effective in modeling the true impacts of toxicant exposure on human health. Agonist-induced platelet activation and aggregation may vary from species to species, in part, by their mechanism of action or potency. Studies involving human platelets may differ from animal-based platelet studies because: 1) human-based studies reflect variation due to a person's way of life and diet as well as use of certain medications, e.g., aspirin, that

influence platelet behavior; 2) genotypic differences between species; 3) animal-based studies are controlled: genotype, housing, route and dose of exposure; and, 4) the potency of agonists vary between animals and humans [127, 128]. Thus, a strength of our study is the direct testing of flavorants on human platelets avoiding need of translational inference.

*Wide Range of Common Flavorants Tested:* Our study uses common flavorants found in thousands of e-cig flavoring profiles currently on the tobacco market [92, 129-131]. The diversity of flavor profiles continues to increase over time to attract new e-cig users [116]. Our study investigates the effect of pure flavorants on platelet aggregation rather than the effects of flavor profiles that are more chemically diverse, and thus, may exert more complex effects on platelets than the pure compounds used herein.

*Appropriate Test Concentration:* Our series of experiments involved testing effects of flavorants (100  $\mu$ M) on ADP-induced biphasic platelet aggregation. Blood levels of flavorant compounds are unreported. To justify levels of flavorants used in this study, we estimated potential blood levels based on reported concentrations of common flavorants in e-cig liquids (**Table 3**).

Using the highest reported e-liquid concentrations in **Table 3**, we estimated the blood levels of flavorants after vaping using convenient assumptions, e.g., 100% efficiency in both transfer from e-liquid to aerosol and from aerosol to the blood (**Table 3**). During e-cig use, e-liquid consumption is minimally dependent on user topography and e-cig power settings, and so e-liquid consumption will vary in different studies. Although the volume of e-liquid aerosolized in any puff depends on several factors (e.g., power, puff volume, puff duration, etc...), we used 2  $\mu$ L as a conservative estimate of e-liquid consumed during

a 100 mL puff [132, 133]. This is based on: 1) the literature [133]; 2) our experience in an experimental setting wherein a 91.1 mL puff consumed 4.0-6.9 mg of e-liquid, which is more volume than we use in the calculation [65]; and, 3) because we previously used this same volume in estimates of cinnamaldehyde uptake [132]. Moreover, as pulmonary blood first receives the compounds of inhaled e-cig aerosols, we chose a modest blood volume (100 mL; 2 heart beats worth) for dilution of pure flavorants. Using cinnamaldehyde as our example flavorant with a reported concentration range of 0.8 – 155 mg/mL (**Table 3**), 2  $\mu$ L of aerosolized e-liquid [155 mg/mL] contains 0.31 mg cinnamaldehyde. Per our assumptions, 0.31 mg cinnamaldehyde is absorbed into blood, i.e., 100 mL blood = 0.31 mg/dL. Thus, under these conditions, blood cinnamaldehyde concentration would be approximately 23.46  $\mu$ M. Of course, we appreciate that whole-body dilution will occur, yet not instantaneously. Moreover, platelet aggregation is a rapid event, and thus, a thrombus could form rather quickly upon activation. We chose a relatively high level of pure flavorant (100  $\mu$ M) to test this idea *in vitro*, and more critically to not miss a toxicological threshold of these common parent flavorants. Additionally, during active e-cigarette use, flavorants may accumulate in blood for many reasons. Notably, most of these common flavorant compounds are GRAS, yet this designation is for their ingestion, and toxicity data regarding their inhalation is not available. Finally, the thermally-derived products of flavorants after heating need to be better characterized and tested for their effects on platelets.

*Strong Positive Control Flavorant:* Clove oil has been demonstrated as a potent antithrombotic agent of platelet aggregation [134, 135]. Eugenol as a major constituent of clove oil is also well-documented for having potent anti-platelet aggregation effects

[136]. In human PRP, eugenol significantly inhibits arachidonic acid- (AA; 0.8 mM), collagen- (20 µg/mL), and platelet activating factor- (PAF; 0.8 µM) induced aggregation [137]. In our current study, we tested the direct effects of eugenol on ADP-induced human platelet aggregation. We found that eugenol at a concentration as low as 10 µM inhibits ADP-induced aggregation and does so solely via inhibition of phase 2 – a step that is dependent on platelet secretion of granules and platelet recruitment. This finding is consistent with the known mechanism of clove and eugenol suppression of platelet action that is similar to aspirin and other inhibitors of prostaglandin biosynthesis, e.g., thromboxane A<sub>2</sub> (TxA<sub>2</sub>) [136-138]. In this study, eugenol proved useful as a strong positive control flavorant that directly inhibits ADP-induced platelet aggregation, and these data confirm that proper testing conditions were used for isolated human platelets.

### ***Limitations of Study***

This study also has a few limitations: 1) only one agonist (ADP) was tested; 2) contrasting results with literature; and, 3) parent flavorants vs. flavorant thermal degradation products.

Use of ADP agonist: We did not test for effects of flavorants on other agonists of platelet aggregation (e.g., thrombin, TxA<sub>2</sub>, serotonin, collagen, AA, or epinephrine) because these agonists, with the exception of epinephrine, do not induce a biphasic platelet response [139]. The biphasic response of ADP provides an opportunity to reveal mechanisms by which flavorants, in combination with ADP, act on platelets. Thus, we chose ADP as the ‘best option’ of all agonists for this study because it stimulates a biphasic response [140]. We recognize that other agonists reveal other pathways of platelet

aggregation that may become activated (or inhibited) upon exposure to a flavorant, which are also important to study.

Comparisons with literature: Our study found no effects of common e-liquid flavorants (excepting for our positive control eugenol) on ADP-induced platelet aggregation, although this general finding is contradicted by a few published studies. For example, several flavorants that we tested, cinnamaldehyde, vanillin, menthol, and menthone, are reported to have anti-platelet activity. For example, Takenaga et al. finds that cinnamaldehyde (300-1,000  $\mu\text{M}$ ) inhibits ADP (2  $\mu\text{M}$ )- and collagen (0.5  $\mu\text{g/mL}$ )-induced human platelet aggregation via suppressing release of AA and TxA<sub>2</sub> and B<sub>2</sub> in platelet granules [141]. In rat PRP, cinnamaldehyde (150-600  $\mu\text{M}$ ) also decreases collagen- (100  $\mu\text{g/mL}$ )- and thrombin- (3 U/mL) induced platelet aggregation [142]. Vanillin (100 ng/mL, 0.657  $\mu\text{M}$ ) suppresses AA- (100  $\mu\text{M}$ ) induced rabbit platelet aggregation [143]. To our knowledge, no other study using vanillin was conducted in human PRP with ADP as the agonist. Both menthol (5 mM) and menthone (5 mM) inhibit collagen- (5  $\mu\text{g/mL}$ ) and ADP- (5  $\mu\text{M}$ ) induced human platelet aggregation *ex vivo* [144], however, again the level used (5 mM) is an order of magnitude higher than both our estimated blood level and our test concentration of 100  $\mu\text{M}$ . Last, limonene (500  $\mu\text{M}$ ; 5x our test [100  $\mu\text{M}$ ]) inhibits thrombin-induced platelet activation via activation of the adenosine A<sub>2A</sub> receptor [145]. Despite apparent contradictions between our findings and published studies, differences in effects of parent flavorants appear attributable to use of higher concentrations and different agonists of platelet aggregation.

Flavorant thermal degradation products: E-cigs heat their liquids that can result in thermal degradation of parent flavorants. Although we tested 15 common flavorants in e-

liquids representing seven distinct chemical classes, we did not test the variety of compounds formed within and during the heating of e-liquids. For example, flavor acetals or thermally-derived flavor products that result from chemically altering the parent flavorants and forming chemical adducts during heating of e-cig solvents or that appear as stable adducts with propylene glycol (PG) and vegetable glycerol (VG) at room temperature, were not tested [69]. Interestingly, some acetal adducts are more toxic than their parent flavorants in lung epithelial cells [146]. The effect of flavor acetals on platelet function have not been studied. As such, we did not address the effects of flavorant thermal degradation products or PG/VG acetals on platelet aggregation because establishing a baseline of the effects of pure flavorant compounds, which can likely reach [ $\mu\text{M}$ ] levels in the pulmonary blood (see **Table 3**), on human platelets is needed.

## **SUMMARY AND CONCLUSION**

Under the conditions tested, our range of common e-cig fluid flavorants had limited direct effect on isolated human platelets aggregation stimulated by ADP. As discussed, flavorants may have agonist-dependent effects, e.g., only augment platelet activation of specific agonists such as collagen, thrombin, etc..., however, past reports used incredibly high levels of flavorants that are not justified relative to e-cigarette use. Additionally, it is important to note that e-cig products are heated instruments. The chemical structure of flavorants can be altered when heated, and thus, these new compounds (e.g., flavor acetals) may impact platelet behavior. In fact, some flavorant thermal degradation products are more potent biologically than their parent compounds [146]. To address this gap in knowledge, future studies should assess the direct effects of products of heated flavorants on agonist-induced human platelet aggregation.

## CHAPTER III

### EFFECT OF INHALED TOBACCO-DERIVED AEROSOLS AND ALDEHYDES ON PLATELET ACTIVATION *IN VIVO*

#### INTRODUCTION

Exposure to tobacco smoke is associated with onset of cardiovascular disease [147]. Thrombosis and platelet activation are common pathologies of cigarette smoke exposure leading to CVD: such as hypertension [148], stroke [149], and thromboembolism [150]. Furthermore, the risk of causing platelets to activate is increased with frequent use of cigarettes [151]. Smoking has long been associated with increased response to ADP-induced platelet-platelet aggregation [152, 153]. Though platelet aggregation is one indicator of adverse platelet function, there are additional inflammatory and thrombogenic biomarkers of harm associated with smoking.

Platelets have been implicated as mediators of inflammatory responses through various mechanisms [154]. Activated platelets not only bind to endothelium and to other platelets, but they also bind to leukocytes [155-157] and form complexes called platelet-leukocyte aggregates (PLAs). PLAs are an integral part of hemostasis and blood clots, and these are also biomarkers of CVD [158]. These aggregates are also important in leukocyte recruitment to the site of vascular injury, thus contributing to inflammatory responses [159]. A study shows that the presence of PLAs within the circulatory system are increased in patients with acute coronary disease [160]. Acute exposure to cigarette smoke increases formation of PLAs in hamsters [161]. Acute exposure to MCS-derived aldehyde constituents, specifically inhaled acrolein, significantly increase platelet-



monocyte aggregates, a subset of PLAs, in C57BL/6 male mice compared with mice exposed to filtered air [77]. In the presented studies, I evaluated the effects of MCS, e-cig aerosol, and aldehyde exposures on PLAs *in vivo*.

Platelet factor 4 (PF-4) is another marker of platelet activation. When an activated platelet undergoes degranulation, PF-4, an abundant chemokine stored within the  $\alpha$ -granules, is expressed on the platelet surface. PF-4 is critical in promoting blood coagulation [162] and is a chemoattractant to leukocytes such as neutrophils [154]. PF-4 plays a major role in neutralizing of heparin-like molecules in the plasma and on the endothelial surfaces of blood vessels, thus impeding antithrombin activity and inducing clotting [163, 164]. Plasma PF-4 levels are elevated in smokers [36, 165, 166] and in mice acutely exposed to oral or inhaled acrolein [77]. In the presented studies, I evaluated the effects of tobacco-derived aerosols on plasma PF-4 levels *in vivo*.

It is clear that platelet activation is a biomarker of harm due to cigarette exposure. Researchers have inferred and shown that tobacco-derived aerosols influence platelet activation in other ways than aggregation. Here, I have evaluated the effects of tobacco-derived aerosols and their constituent aldehydes on markers of platelet activation and thrombosis: PLA formation and plasma PF-4 levels.

## MATERIALS AND METHODS

### *Materials*

Reagent-grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

### *Methods*

Mice: As described in Conklin *et al.* 2017 [167], we obtained C57BL/6J male and female mice from The Jackson Laboratory (Bar Harbor, ME). All mice were treated according to the Guiding Principles for the Care and Use of Animals in Research and Teachings as adopted by the American Physiological Society, and all protocols were approved by the University of Louisville Institutional Animal Care and Use Committee. Before and after exposures, the mice were housed under pathogen-free conditions in the University of Louisville vivarium under controlled temperature and 12 h light: 12 h dark cycle. Mice were maintained on a standard chow diet (Rodent Diet 5010, 4.5% fat by weight, LabDiet; St. Louis, MO).

MCS Exposure System: Naïve mice were exposed to MCS (50% of smoke of 12 KY Reference 3R4F cigarettes/6h) as previously described in Conklin *et al.* 2018 [65]. A software-controlled (FlexiWare) cigarette-smoking robot (CSR) (SCI-REQ; Montreal, Canada) system regulated the mechanical generation of aerosols from these cigarettes.

E-cig Aerosol Exposure System: As described in Jin *et al.* 2021 [168], a software-controlled (FlexiWare) cigarette-smoking robot (CSR; SCI-REQ; Montreal, Canada) system was used in the mechanical generation of aerosols from PG:VG mixtures. To control the generation of volatile organic compounds (VOCs) in e-cig aerosols, we used a

defined e-cig platform. PG:VG mixture (30:70 ratio, vol/vol) was loaded into a refillable, clear tank atomizer with a fixed coil resistance (Mistic Bridge; ~3.0 ohm; purchased online) coupled with a rechargeable bluPLUS+ (3.7 V) battery (power out-put of ~8W). The atomizer tank was weighed before and after use to quantify solution consumption (g/puff). A 9-min session was composed of 18 puffs (4 s/puff, 91.1 mL/puff, 2 puffs/min). For the exposures, 20 sessions were evenly spaced over a 6h exposure per day for 4 consecutive days. Total suspended particulate (TSP) matter was monitored in real time with an inline infrared Microdust Pro 880 nm (Casella) positioned upstream of the exposure chamber (5 L, SCI-REQ).

*Acrolein and Crotonaldehyde Exposures:* To parallel our previous study of chronic (12 weeks, 1 ppm) acrolein (AC) exposure in mice [167], naïve mice were exposed to HEPA- and charcoal-filtered air, AC at 1 ppm, or crotonaldehyde (CR) (1 or 3 ppm, 6h/d) for 4 consecutive days or 12 weeks (1 ppm; 6h/d, 5 d/week) using a custom exposure system and a certified permeation tube (Kin-Tek; LaMarque, TX).

*Formaldehyde and Acetaldehyde Exposures:* For formaldehyde (FA) and acetaldehyde (AA) exposures, naïve mice were exposed to either HEPA- and charcoal-filtered air, FA, or AA for 4 consecutive days (5 ppm, 6 h/day) using an exposure system equipped with a certified permeation tube in a calibrated heating oven (Kin-Tek, LaMarque, TX) as described in Lynch *et al.* 2020 [81]. Levels of AA were monitored real time with an inline photoionization detector (isobutylene calibration), whereas levels of FA were monitored real-time with an inline electrochemical sensor (CO calibration) (MultiRAE Pro, RAE Systems, Burlington, VT). Both gases were monitored upstream of the exposure chamber (30-liter, flow 7lpm).

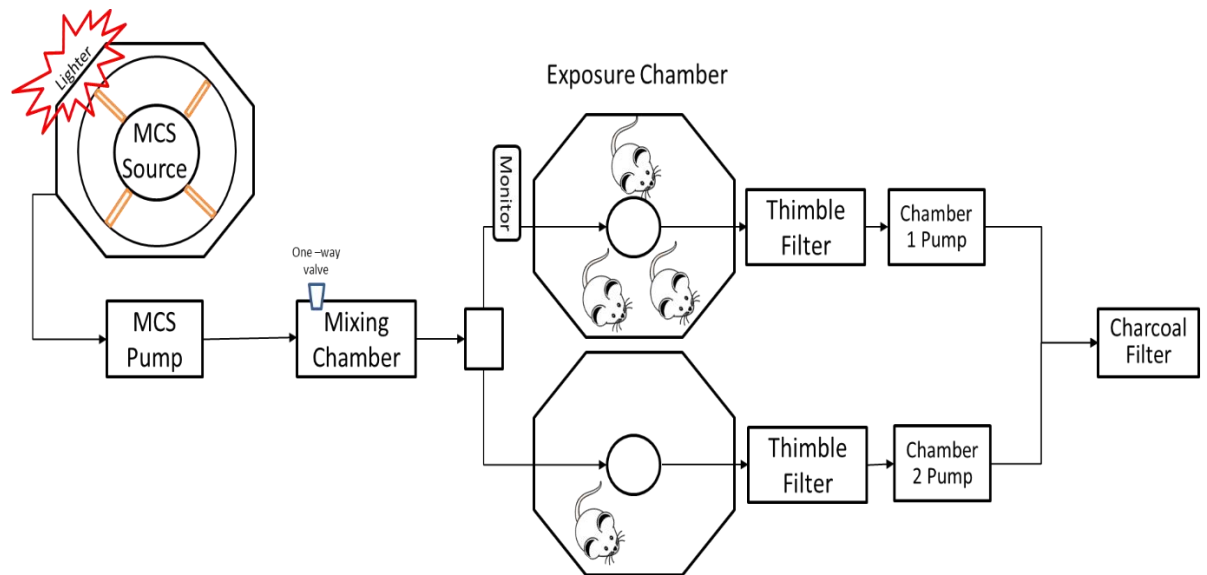
All whole-body exposures were done between 7:00 AM and 2:00 PM in the absence of food or water. Immediately following the final exposure, mice were euthanized by sodium pentobarbital ( $\approx 150$  mg/kg, i.p.) and ventral thoracotomy and exsanguination with cardiac puncture for peripheral blood collection in EDTA-coated syringes.

*Platelet-Leukocyte Aggregates (PLAs)*: After the final exposure, the mice were immediately euthanized and peripheral blood collected and used to detect PLAs. PLAs were identified by flow cytometry and quantified as events double positive for CD41 (platelets) and CD45 (leukocytes) as previously described in Conklin *et al.* [167] with slight modifications. Briefly, aliquots of whole blood were diluted (1:4) with HEPES-Tyrode solution before fixation [paraformaldehyde, Fc 1.6%, room temperature (RT) for 30 mins]. Red blood cells were lysed (2mL MilliQ water), and the sample was centrifuged (400g, 5 min, RT). The sample pellet was resuspended and incubated with 1% Fc Block (5 $\mu$ L; 10 min) before staining for 30 min with FITC-labeled anti-CD41 and APC-labeled anti-CD45 or isotype-matched negative controls (FITC-IgG1; APC-IgG2b $\kappa$ ). Stained cells were washed with HEPES-Tyrode solution containing 1% BSA, centrifuged at 400g for 5 min, and resuspended in HEPES-Tyrode solution (250 $\mu$ L). A BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA) was used to analyze stained cells; a minimum of 20,000 events was collected for each sample.

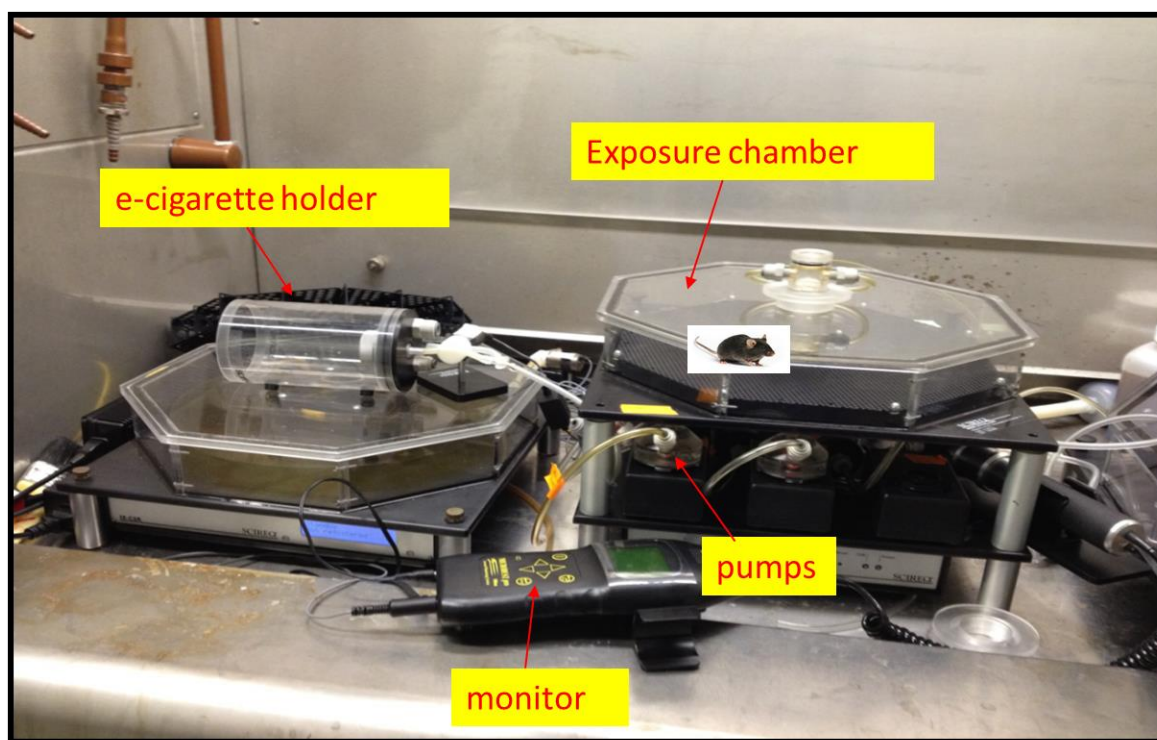
*Platelet Factor 4 (PF-4) Assay*: As described by Sithu *et al.* [77], plasma PF-4 levels were assayed by a sandwich ELISA using DeoSet Mouse PF-4/CXCL4 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, 96-well ELISA plates were coated with a rat anti-mouse PF-4 capture antibody (2 $\mu$ g/mL in PBS) for 16h-overnight at RT. Wells were washed free of the unbound antibody and

blocked with 1% BSA for 1h at room temperature. Plasma samples or the PF-4 standards were incubated in the coated wells for 2h at RT. Wells were washed three times and then incubated with a biotinylated goat anti-mouse PF-4 antibody (100ng/ ml) for 2h at room temperature. Wells were washed again and then incubated with streptavidin conjugated to horseradish-peroxidase for 20 min at RT. The washing step was repeated and the substrate, tetramethylbenzidine (TMB) was added and the reaction was incubated for 15 min. The reaction was stopped with 1N H<sub>2</sub>SO<sub>4</sub> and the developed color was measured using a microplate reader at 450 nm.

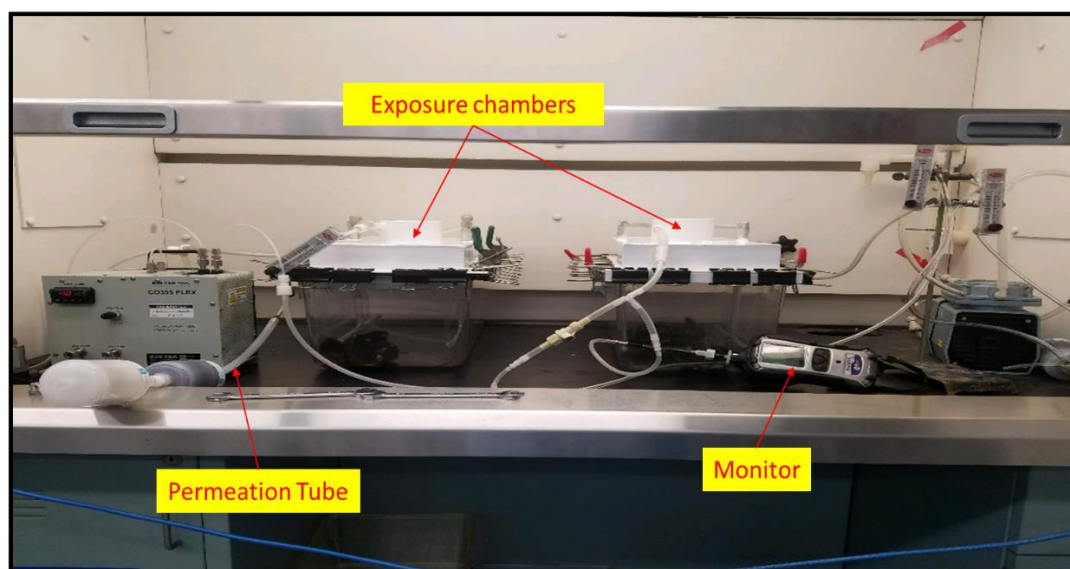
*Statistical Analysis:* Data are presented as means  $\pm$  standard error (SE). For statistical comparison between two groups, the Mann-Whitney U test was used (SigmaPlot ver.12). Significance was accepted where  $p < 0.05$ .



**Figure 6.** MCS inhalation exposure system. The MCS exposure involves a CSR lighting 12 3R4F reference tobacco cigarettes, and the smoke is then pumped and transferred into the exposure chambers that house the mice. This follows an ISO protocol (i.e., 2 s puff, 35 mL puff, 1 puff/min, 9 puffs/cigarette; 2 cigarettes/h [65]). The total suspended particulate (TSP) matter was monitored in real time with an inline infrared forward scattering monitor (MicroDust Pro; Casella CEL Ltd., Bedford, UK). (This schematic was drawn by Whitney Theis, M.S.P.H.)



**Figure 7.** E-cig inhalation exposure system. The pump draws out vapor from the e-cig and transfers the vapor into the exposure chamber in which the C57BL/6 male mice are contained during exposure.

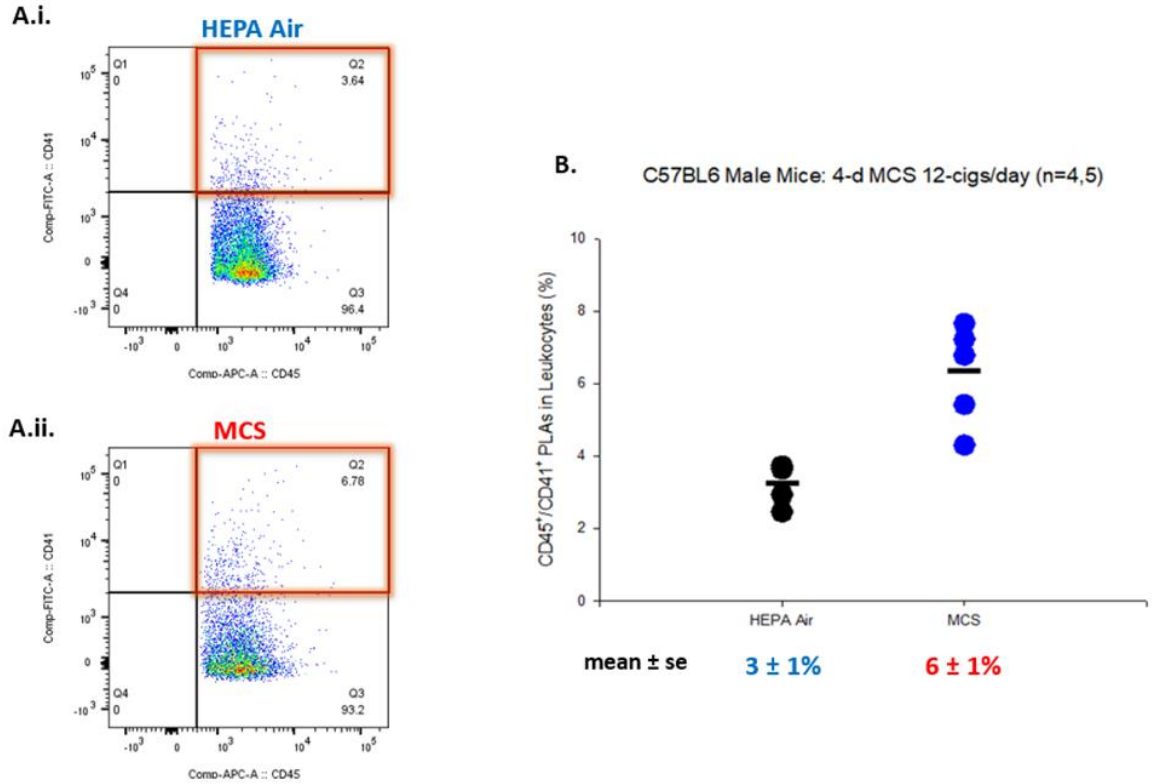


**Figure 8.** Aldehyde inhalation exposure system. C57BL/6 male or female mice are placed inside a sealed exposure chamber. Steady, continuous 6h flow of aldehyde gas is pumped via certified permeation tubes (Kin-Tek; LaMarque, TX) into the chamber per specified duration in each study [111, 169]. During exposure, aldehydes are monitored continuously with an in-line calibrated photoionization detector (PID; ppbRAEPlus, Rae Industries, Sunnyvale, CA) [111].



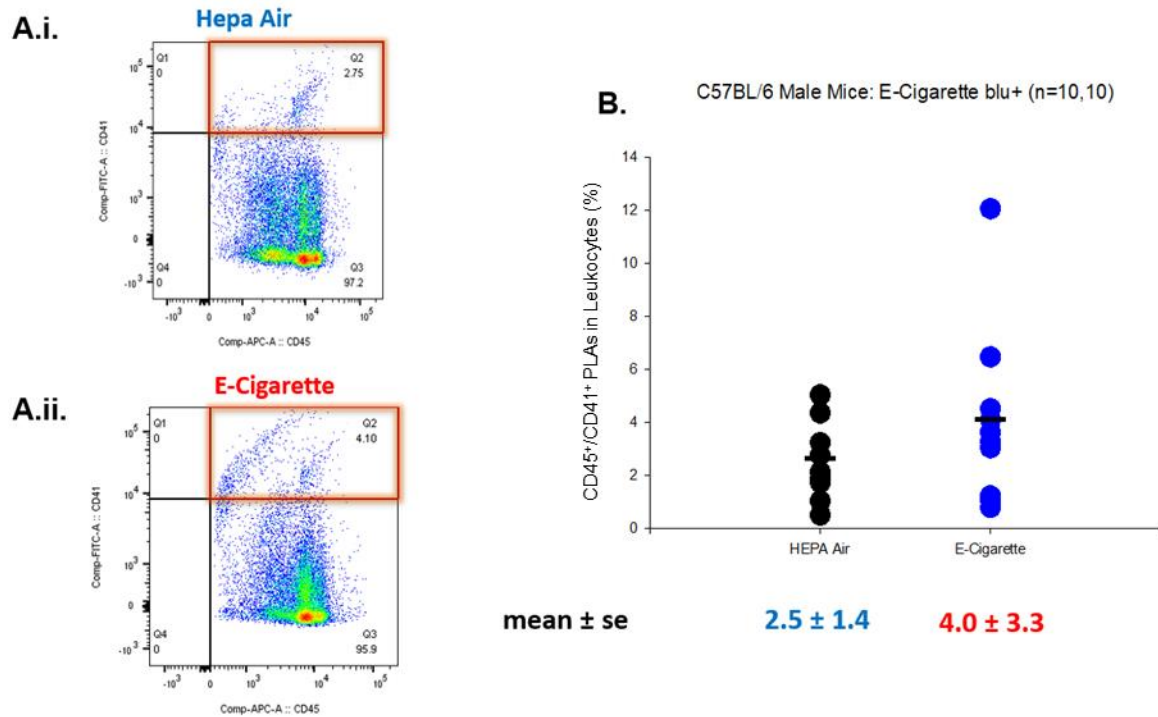
## RESULTS

**A. Effect of acute MCS exposure via inhalation on C57BL/6 male mice.** Various pathophysiological mechanisms have been proposed for tobacco smoke-induced CVD. Salahuddin *et al.* proposed that cigarette smoke is comprised of oxidant gases and toxic chemicals that inevitably release and activate free radicals that cause oxidative stress, increase inflammation, and decrease NO production [170]. As a result of these effects, platelet activation and thrombosis are more likely to occur. With this knowledge, I hypothesized that acute exposure to MCS will lead to platelet activation *in vivo* as measured by increased formation of PLAs. To test this hypothesis, C57BL/6 male mice were exposed to MCS (12 cigs/d, 4d) or HEPA-filtered air (control; 4d, 6h/d). Immediately after final exposure, the mice were euthanized and their peripheral blood was collected and prepared for flow cytometric analyses for identification and quantification of CD45<sup>+</sup>/CD41<sup>+</sup> PLAs. My results indicate that acute exposure of mice to MCS significantly increased PLA formation compared with air control (**Figure 9**). These data suggest that exposure to MCS causes platelet activation.



**Figure 9.** Effects of acute MCS exposure on PLAs in C57BL/6 male mice. Flow cytometry analysis was performed in order to identify CD45<sup>+</sup>/CD41<sup>+</sup> PLAs in 100μL male mouse blood following an acute 4d exposure to MCS (50% of smoke from 12-cigs / 6h; n=4,5). Representative flow cytometry dot plots of (**Ai.**) control (HEPA-filtered air) versus (**Aii.**) MCS-exposed mice are displayed. (**B**) Summary data displays each individual mouse exposed to either air or MCS. PLAs are characterized as 20,000 CD45/CD41 double positive events, located in quadrant 2 (Q2). The mice exposed to MCS showed significant increase in PLAs compared with control p=0.016 via Mann-Whitney U test.

**B. Effect of acute e-cig aerosol exposure via inhalation on C57BL/6 male mice.** As previously mentioned and shown in Conklin *et al.* 2018 [65], we know that MCS and e-cig aerosols have common aldehyde constituents. Therefore, in this study, I investigated if e-cig exposure induces platelet activation similar to that observed in our MCS study. I hypothesized that acute exposure of mice to e-cig will lead to platelet activation *in vivo* as assessed by increased formation of PLAs. C57BL/6 male mice were exposed to e-cig aerosol (blu+, Classic Tobacco flavor; 4d, 6h/d) or HEPA-filtered air (control; 4d, 6h/d). Immediately after the final exposure, the mice were euthanized, and their peripheral blood was collected and prepared for flow cytometric analyses of CD45<sup>+</sup>/CD41<sup>+</sup> PLAs. My results indicate no change in PLA formation between e-cig exposed mice compared with the air control mice (**Figure 10**).



**Figure 10.** Effects of acute e-cig exposure on PLAs in C57BL/6 male mice. Flow cytometry analysis was performed in order to identify CD45<sup>+</sup>/CD41<sup>+</sup> PLAs in 100μL male mouse blood following an acute 4d exposure to e-cig aerosol (blu+ Classic Tobacco; 6h/d; n=10,10). Representative flow cytometry dot plots of (**Ai.**) control (HEPA-filtered air) versus (**Aii.**) e-cig-exposed mice are displayed. (**B**) Summary data displays each individual mouse exposed to either air or e-cig aerosol. PLAs are characterized as 20,000 CD45/CD41 double positive events, located in quadrant 2 (Q2). The mice exposed to e-cig aerosol showed no difference in PLAs compared with control, p=0.273 via Mann-Whitney U test.

### **C. Effect of acute exposure to PG:VG and acrolein via inhalation on PLAs in**

**C57BL/6 male and female mice.** In addition to nicotine, PG and VG are in e-liquids common in e-cigs. Both of these compounds are listed as generally regarded as safe (GRAS) for oral consumption by the U.S. Food and Drug Administration (FDA) [171]. However, exposure by inhalation is associated with toxicities. Inhaled PG is associated with upper respiratory irritation, asthma [172], and pneumonia [173, 174]. Inhaled VG is associated with metaplasia and irritation to the upper respiratory tract. [175]. When heated, as occurs during use of e-cigs, PG and VG generate toxic aldehydes. VG-derived AC exposure in male mice via inhalation induces platelet activation and increased platelet-monocyte aggregate formation compared with mice exposed to filtered air [77]. In this study, I investigated if exposure of mice to PG:VG-derived aerosols or to AC vapor induces platelet activation. I hypothesized that acute exposure to these aerosols and vapors would lead to platelet activation *in vivo* as assessed by increased formation of PLAs. C57BL/6 mice were exposed to PG:VG (30:70 ratio) or AC (1 ppm) aerosols (4d, 6h/d) or HEPA-filtered air (control; 4d, 6h/d). Immediately after the final exposure, the mice were euthanized, and their peripheral blood was collected and prepared for flow cytometric analyses of CD45<sup>+</sup>/CD41<sup>+</sup> PLAs. My results indicate a significant increase in PLAs due to exposure of female mice to PG:VG-derived aerosols compared with control. (**Table 4**). All other PLA results are insignificant.

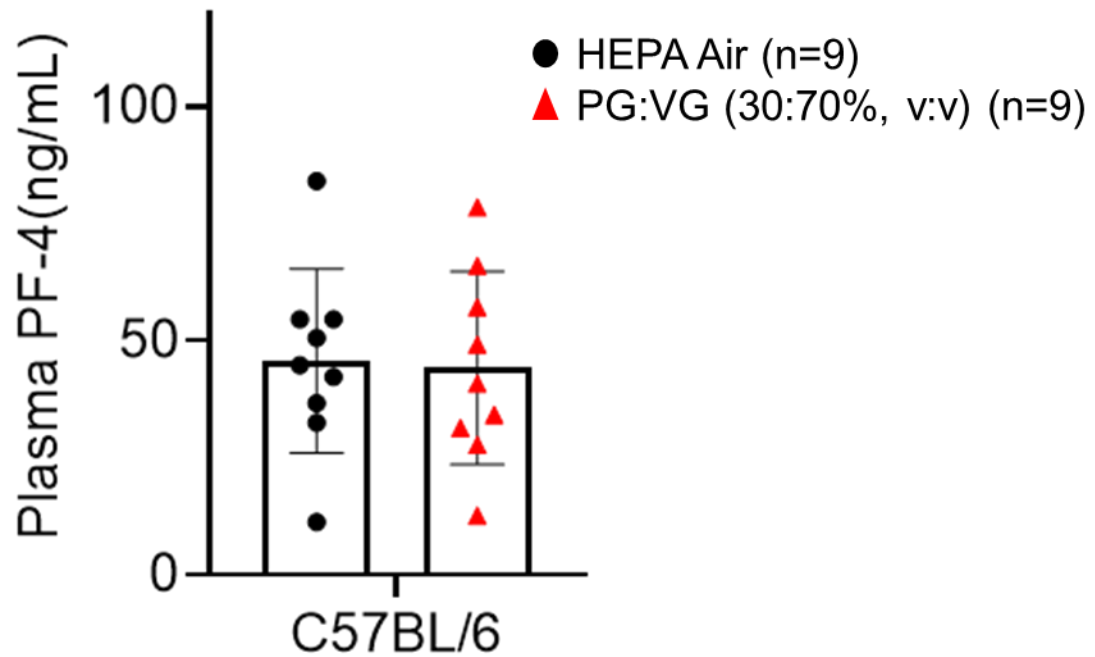
**Table 4.** PLA (%) changes in C57BL/6 male and female mice acutely exposed to air, PG:VG (30:70%), or acrolein (AC). Flow cytometry analyses were performed in order to identify CD45<sup>+</sup>/CD41<sup>+</sup> PLAs in 100µL male mouse blood following exposure to air, PG:VG (30:70), or AC (1 ppm). PLAs are characterized as 20,000 CD45/CD41 double positive events, located in quadrant 2 (Q2).

	<b>Exposure (6h/d; 4d)</b>		
	<b>Air Control</b>	<b>PG:VG (30:70%)</b>	<b>AC (1 ppm)</b>
<b>Male PLAs (% leukocytes)</b>	<b>6.0 ± 1.0</b>	<b>5.7 ± 1.1</b>	<b>7.4 ± 0.7</b>
<b>Female PLAs (% leukocytes)</b>	<b>5.6 ± 2.5</b>	<b>12.6 ± 2.1*</b>	<b>7.5 ± 1.5</b>

Values are means ± SE; n = 4-25 mice per group. Abbr.: PG; propylene glycol; PLAs, platelet-leukocyte aggregates; VG, vegetable glycerin.

**D. Effects of acute exposures to PG:VG and AC via inhalation on plasma PF-4 levels in C57BL/6 male and female mice.** Sithu *et al.* acutely exposed C57BL/6 male mice to AC via a whole-body inhalation exposure system and analyzed for PF-4 in plasma via sandwich ELISA. Mice exposed to 5 ppm AC (6h, 1d) had significantly increased PF-4 levels [77]. Mice exposed to 1 ppm AC (6h/d, 4d) also had significantly increased PF-4 levels [77]. Here, I tested for acute (6h/d, 4d) effects of air, PG:VG (30:70), or 1 ppm AC on mouse plasma PF-4 levels. Under the conditions tested, my results indicate no significant changes in PF-4 levels in males exposed to PG:VG (**Figure 11**) while PF-4 levels significantly increased in exposed females (**Figure 12**) compared with respective controls. Our results showed significant increase in PF-4 in male mice exposed to AC compared with control (**Figure 13**). Additionally, the results show no significant changes in plasma PF-4 levels in female mice exposed to AC compared with control (**Figure 14**).

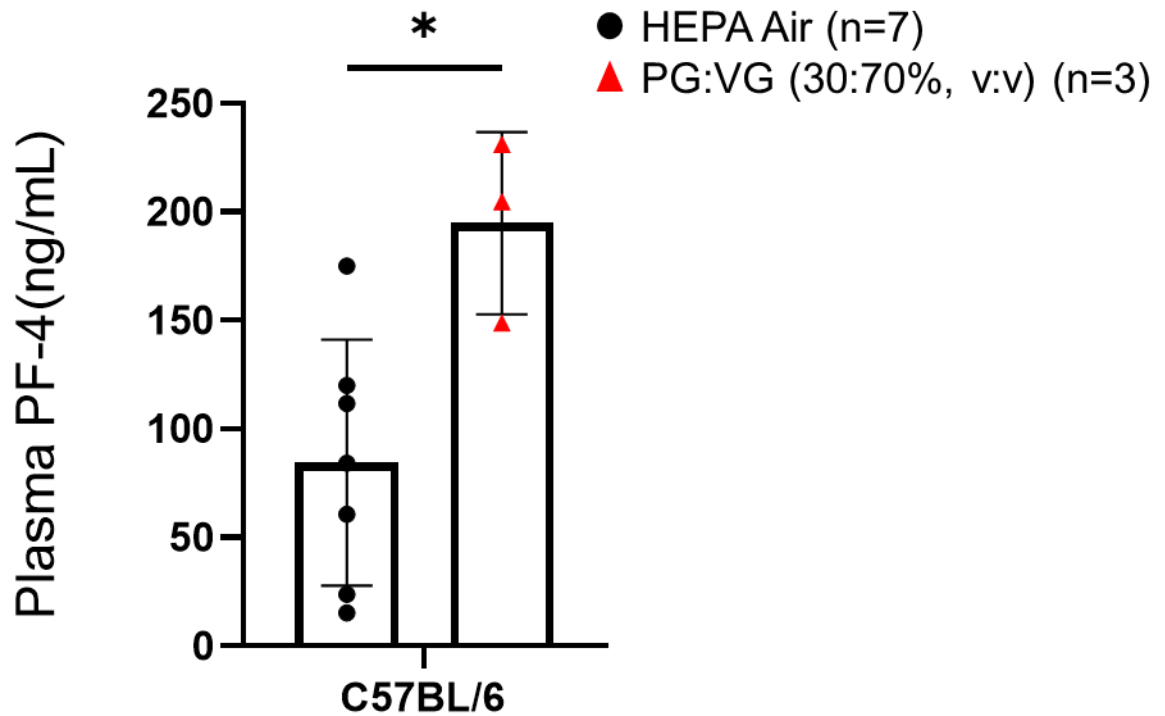
## PG:VG in C57BL/6 Males



**Figure 11.** Effects of PG:VG (30:70) exposure on PF-4 levels in plasma of C57BL/6 male mice. PF-4 in mouse plasma was measured via sandwich ELISA. Mice exposed to PG:VG had no significant difference in PF-4 levels compared with HEPA Air exposed control mice (Mann-Whitney U test).

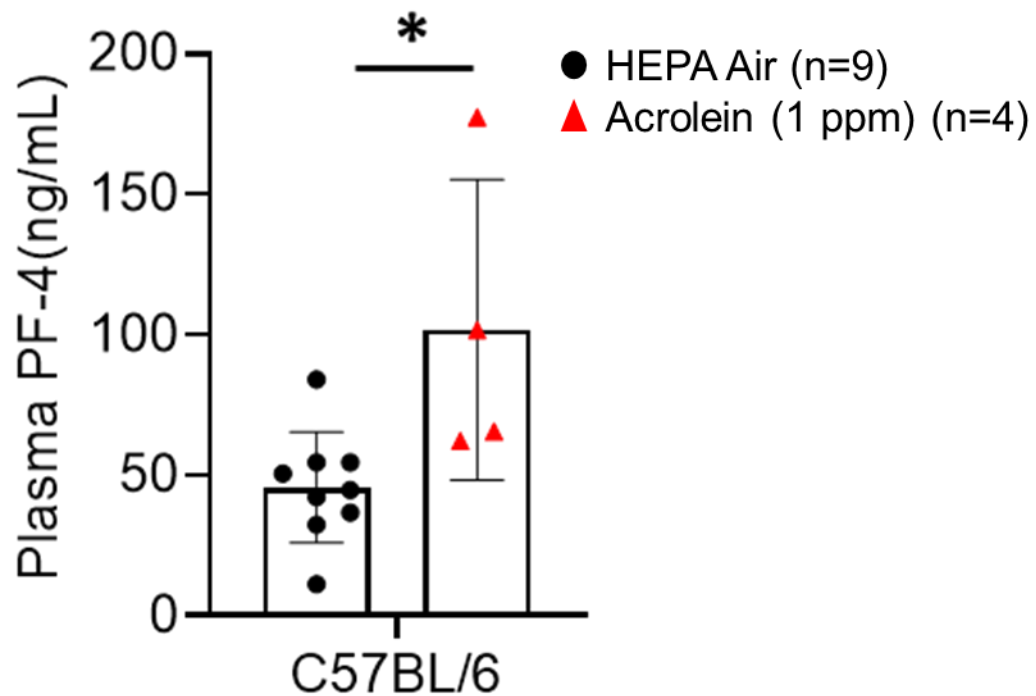


## PG:VG in C57BL/6 Females



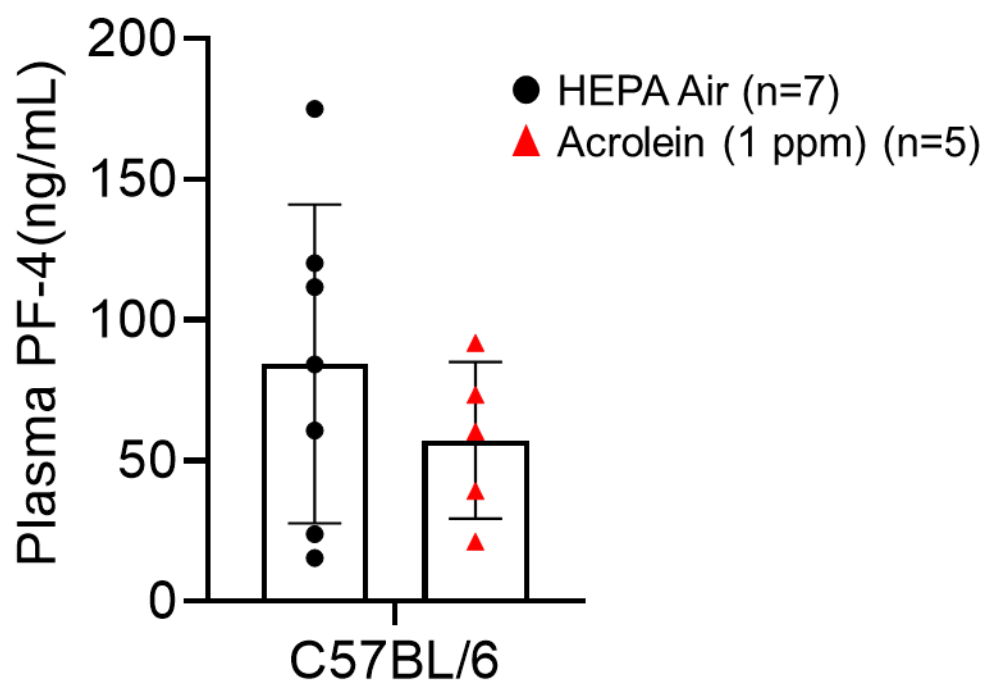
**Figure 12.** Effects of PG:VG (30:70) exposure on PF-4 levels in plasma of C57BL/6 female mice. PF-4 from mouse plasma was measured via sandwich ELISA. PF-4 levels are significantly increased in PG:VG-exposed female mice compared with control (Mann-Whitney U test).

## Acrolein in C57BL/6 Males



**Figure 13.** Effects of acrolein (1 ppm) exposure on PF-4 levels in plasma of C57BL/6 male mice. PF-4 from mouse plasma was measured via sandwich ELISA. The male mice exposed to 1ppm AC showed no significant difference in PF-4 levels compared with control (Mann-Whitney U test).

## Acrolein in C57BL/6 Females



**Figure 14.** Effects of acrolein (1 ppm) exposure on PF-4 levels in plasma of C57BL/6 female mice. PF-4 from mouse plasma was measured via sandwich ELISA. The female mice exposed to 1ppm AC showed no significant difference in PF-4 levels compared with control, via Mann-Whitney U test.

**E. Effects of acute and chronic crotonaldehyde inhalation exposures in C57BL/6 male mice.** Crotonaldehyde (CR) is an understudied toxicant. Both AC and CR are present at high levels in cigarette smoke [176]. My preliminary results indicate an increase in PLA formation in male mice acutely exposed to MCS. AC (1 ppm) exposure in male mice also increases PLA formation [77]. Given that AC and CR are similar compounds in chemical structure, I hypothesized that acute (4d) or chronic (12-wk) exposure to CR will induce platelet activation *in vivo* as assessed by increased formation of PLAs. C57BL/6 male mice were exposed to CR (1 ppm; 6h/d, 4d or 12-wk) or HEPA-filtered air (control; 4d or 12-wk, 6h/d). Immediately after final exposure, the mice were euthanized and their peripheral blood was collected and prepared for flow cytometric analyses of CD45<sup>+</sup>/CD41<sup>+</sup> PLAs. Our results show CR (1 ppm) exposure significantly decreases PLA formation, while CR (3 ppm) exposure increases PLA formation compared with air control [81]. Chronic CR exposure yielded no significant change in PLA formation compared with air control (**Table 5**) [81].

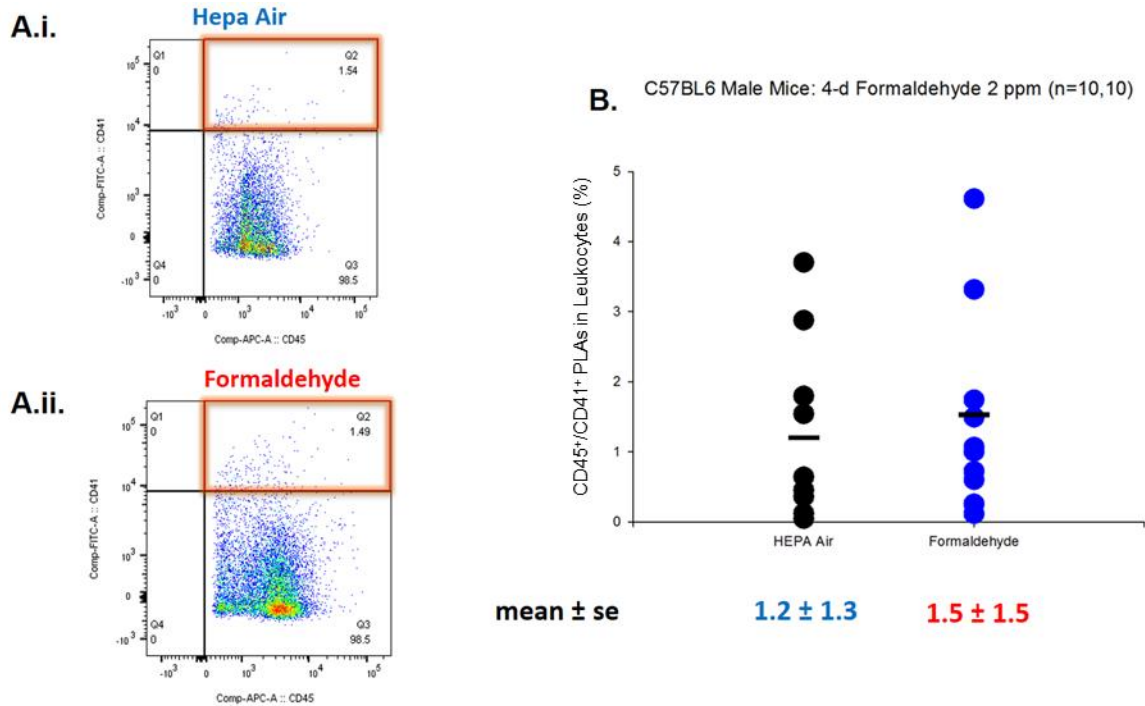
**Table 5.** PLA (%) changes in C57BL/6 male mice acutely (4d) or chronically (12wk) exposed to air or crotonaldehyde (CR). Flow cytometry analyses were performed in order to identify CD45<sup>+</sup>/CD41<sup>+</sup> PLAs in 100μL male mouse blood following exposure to CR (1 or 3 ppm). PLAs are characterized as 20,000 CD45/CD41 double positive events, located in quadrant 2 (Q2). There are significant changes in PLA formation following acute exposure to CR compared with control. There is no change in PLA formation due to chronic CR exposure.

Acute	Exposure (6h/d; 4d)			Reference      [81]
	HEPA-filtered Air	CR (1 ppm)	CR (3 ppm)	
PLAs (%)	6 ± 1	3 ± 0*	12 ± 2*	
Chronic	Exposure (6h/d; 12wk)			
	HEPA-filtered Air	CR (1 ppm)		
PLAs (%)	7 ± 1	9 ± 1		

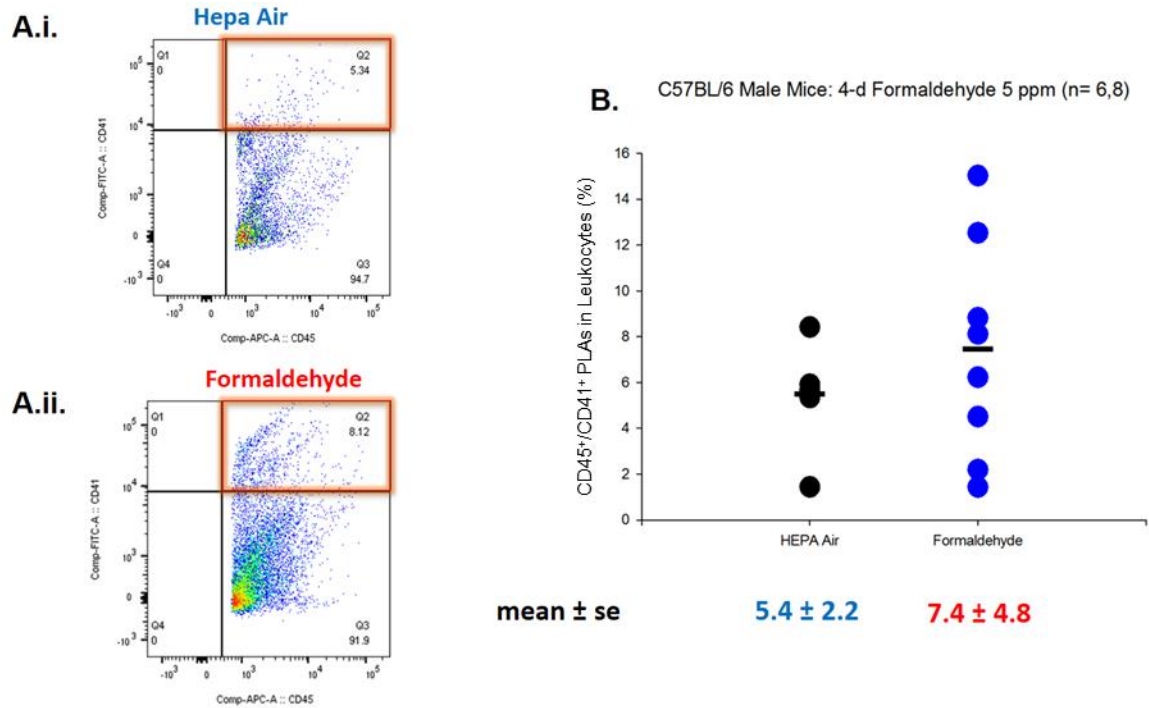
Values are means ± SE; n = 9-15 mice per group. Abbr.: CR: crotonaldehyde; PLAs, platelet-leukocyte aggregates; p<0.05 compared with air control based on Bonferroni's post-test [81].

#### **F. Effect of acute exposure to formaldehyde via inhalation on C57BL/6 male mice.**

In order to assess the effects of formaldehyde (FA) on platelet activation, I measured the concentration- and time-dependent exposure impacts on PLAs. I hypothesized that acute (4d or 2-wk) exposure to FA will induce platelet activation *in vivo* as assessed by increased formation of PLAs. C57BL/6 male mice were exposed to FA (2 or 5 ppm; 6h/d, 4d or 2-wk) or HEPA-filtered air (control; 6h/d, 4d or 2-wk). Immediately after final exposure, the mice were euthanized and their peripheral blood was collected and prepared for flow cytometric analyses of CD45<sup>+</sup>/CD41<sup>+</sup> PLAs. My results indicate that male mice acutely exposed to FA (2 ppm) had no alterations in PLA formation compared with control (**Figure 15**) [168]. Bhatnagar *et al.* found that FA concentrations above 2 ppm increased platelet count [177], I hypothesized that by increasing the concentration, more PLA formation will result. However, treatment with a 5ppm FA still exerted no change (**Figure 16**) [168]. However, the previous report of an increased platelet count resulted from a longer duration (12d) of exposure [177]. Therefore, I completed a two-week exposure to a high concentration of formaldehyde, hypothesizing this would change abundance of PLAs. However, I found no changes in PLA formation compared with air controls (**Figure 17**).

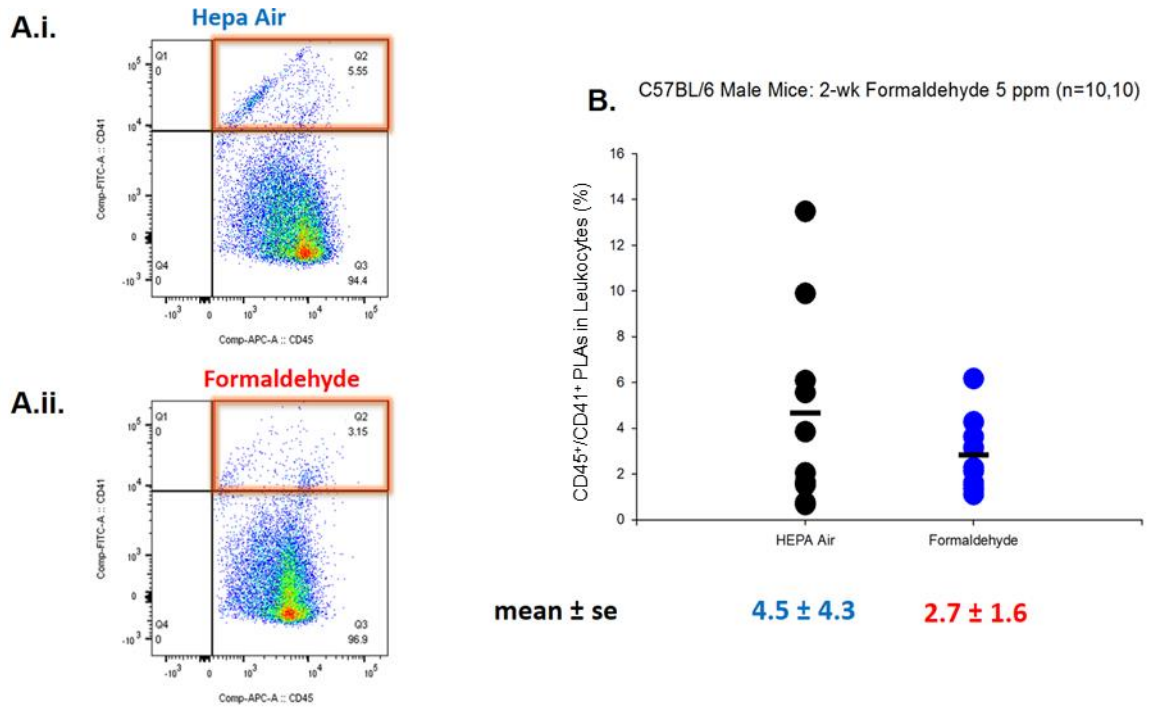


**Figure 15.** Effects of acute formaldehyde (2 ppm) exposure on PLAs in male mice. Flow cytometry analysis was performed in order to identify CD45<sup>+</sup>/CD41<sup>+</sup> PLAs in 100 $\mu$ L male mouse blood following an acute 4d exposure to formaldehyde (6h/d; n=10,10). Representative flow cytometry dot plots of (A.i.) control (HEPA-filtered air) versus (A.ii.) formaldehyde-exposed mice are displayed. (B) Summary data displays each individual mouse exposed to either air or formaldehyde. PLAs are characterized as 20,000 CD45/CD41 double positive events, located in quadrant 2 (Q2). The mice exposed to formaldehyde showed no difference in PLAs compared with control,  $p=0.597$  via Mann-Whitney U test.



**Figure 16.** Effects of acute formaldehyde (5 ppm) exposure on PLAs in C57BL/6 male mice. Flow cytometry analysis was performed in order to identify CD45<sup>+</sup>/CD41<sup>+</sup> PLAs in 100μL male mouse blood following an acute 4d exposure to formaldehyde (6h/d; n=6,8). Representative flow cytometry dot plots of (A.i.) control (HEPA-filtered air) versus (A.ii.) formaldehyde-exposed mice are displayed. (B) Summary data displays each individual mouse exposed to either air or formaldehyde. PLAs are characterized as 20,000 CD45/CD41 double positive events, located in quadrant 2 (Q2). The mice exposed to formaldehyde showed no difference in PLAs compared with control, p=0.368 via Mann-Whitney U test.

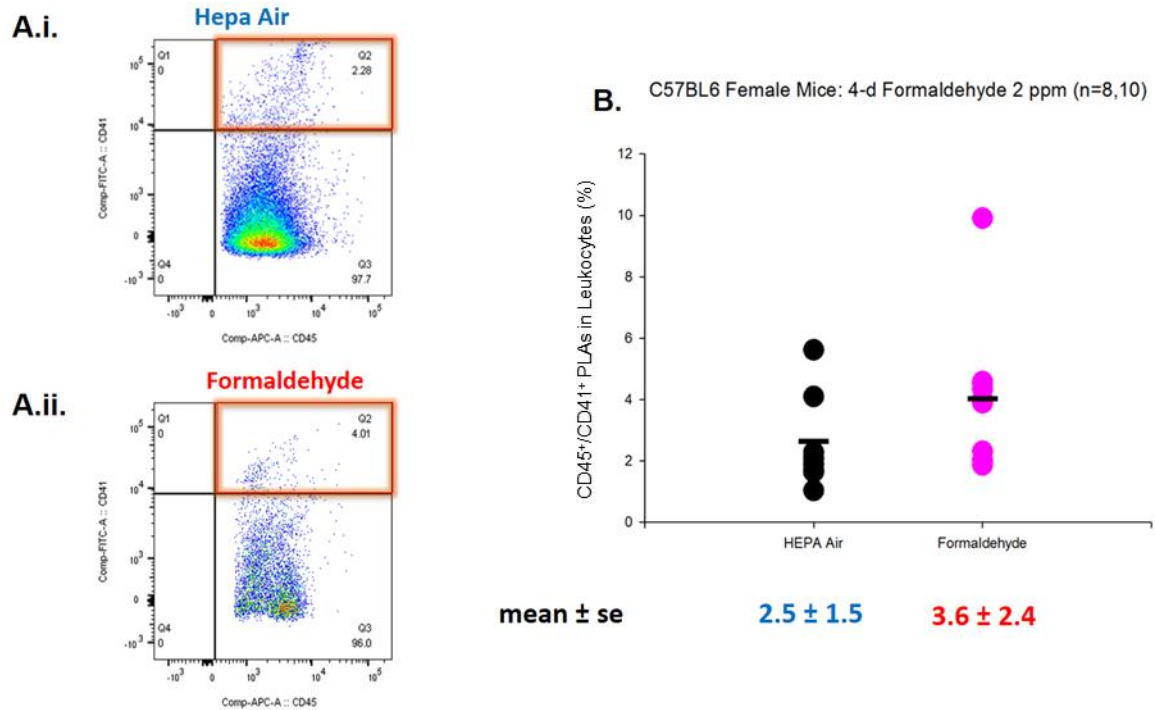




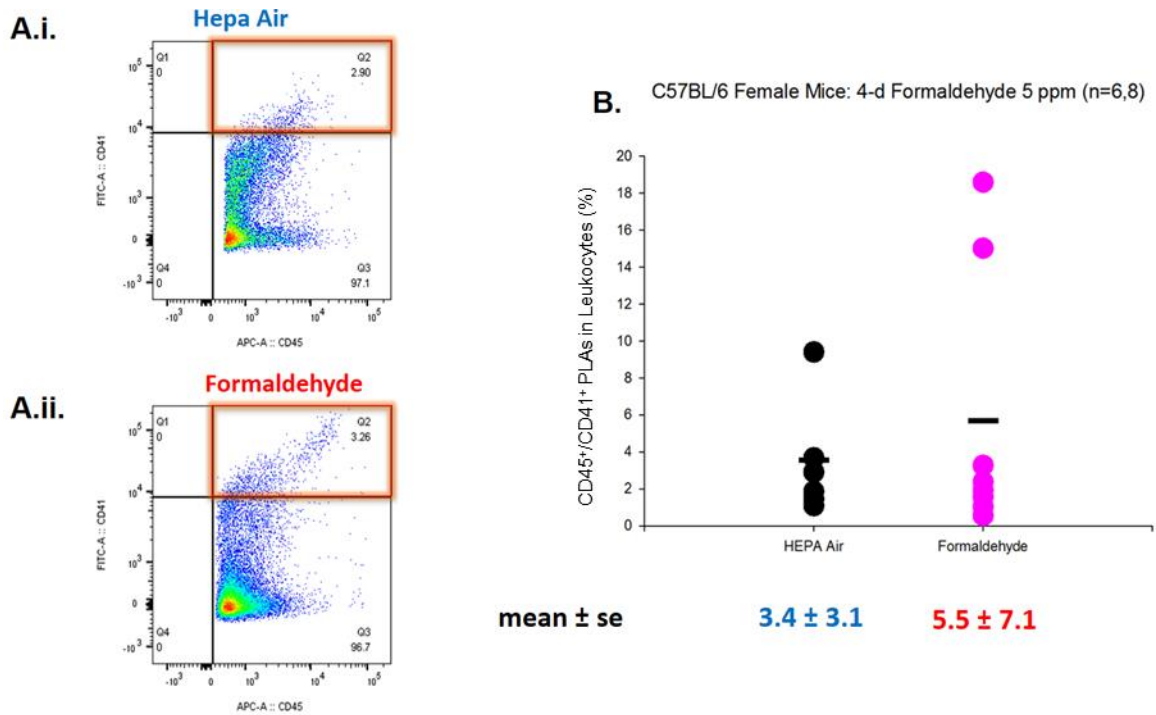
**Figure 17.** Effects of acute (2wk) formaldehyde (5 ppm) exposure on PLAs in C57BL/6 male mice. Flow cytometry analysis was performed in order to identify CD45<sup>+</sup>/CD41<sup>+</sup> PLAs in 100 $\mu$ L male mouse blood following exposure to formaldehyde (6h/d; n=10,10). Representative flow cytometry dot plots of (A.i.) control (HEPA-filtered air) versus (A.ii.) formaldehyde-exposed mice are displayed. (B) Summary data displays each individual mouse exposed to either air or formaldehyde. PLAs are characterized as 20,000 CD45/CD41 double positive events, located in quadrant 2 (Q2). The mice exposed to formaldehyde showed no difference in PLAs compared with control, p=0.678 via Mann-Whitney U test.

### **G. Effect of acute exposure to formaldehyde via inhalation on C57BL/6 female mice.**

Like our previous studies, I measured the effects of FA exposure on PLA outcomes in female mice. I hypothesized that acute (4d) exposure to FA will induce platelet activation *in vivo* as assessed by increased formation of PLAs. C57BL/6 female mice were exposed to FA (2 or 5 ppm; 6h/d, 4d) or HEPA-filtered air (control; 6h/d, 4d). Immediately after final exposure, the mice were euthanized, and their peripheral blood was collected and prepared for flow cytometric analyses of CD45<sup>+</sup>/CD41<sup>+</sup> PLAs. My results indicate that female mice acutely exposed to FA (2 ppm) demonstrated no alterations in PLA formation compared with control (**Figure 18**) [168]. I also increased the concentration of FA, hypothesizing that by increasing the concentration, more PLA formation will result. However, a FA concentration of 5 ppm still exerted no change in PLA abundance (**Figure 19**) [168]. Thus, there were no sex-dependent differences in PLA formation in response to formaldehyde exposure.



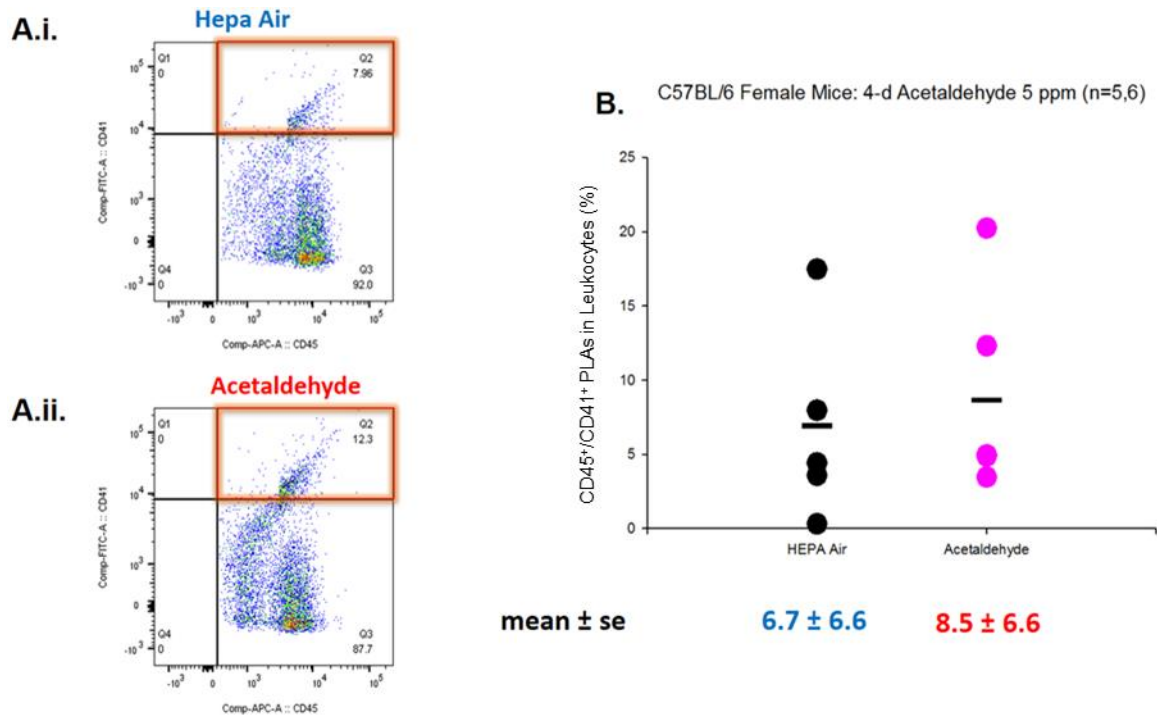
**Figure 18.** Effects of acute formaldehyde (2 ppm) exposure on PLAs in C57BL/6 female mice. Flow cytometry analysis was performed in order to identify CD45<sup>+</sup>/CD41<sup>+</sup> PLAs in 100μL female mouse blood following an acute exposure to formaldehyde (6h/d; n=8,10). Representative flow cytometry dot plots of (A.i.) control (HEPA-filtered air) versus (A.ii.) formaldehyde-exposed mice are displayed. (B) Summary data displays each individual mouse exposed to either air or formaldehyde. PLAs are characterized as 20,000 CD45/CD41 double positive events, located in quadrant 2 (Q2). The mice exposed to formaldehyde showed no difference in PLAs compared with control, p=0.143 via Mann-Whitney U test.



**Figure 19.** Effects of acute formaldehyde (5 ppm) exposure on PLAs in female mice. Flow cytometry analysis was performed in order to identify CD45<sup>+</sup>/CD41<sup>+</sup> PLAs in 100μL female mouse blood following an acute exposure to formaldehyde (6h/d; n=6,8). Representative flow cytometry dot plots of (A.i.) control (HEPA-filtered air) versus (A.ii.) formaldehyde-exposed mice are displayed. (B) Summary data displays each individual mouse exposed to either air or formaldehyde. PLAs are characterized as 20,000 CD45/CD41 double positive events, located in quadrant 2 (Q2). The mice exposed to formaldehyde showed no difference in PLAs compared with control, p=0.503 via Mann-Whitney U test.

#### **H. Effect of acute exposure to acetaldehyde via inhalation on C57BL/6 female mice.**

Acetaldehyde (AA) is a metabolite of ethanol. Investigators have demonstrated that both compounds inhibit platelet aggregation [178, 179]. In order to assess the effects of AA in PLA formation, I performed an exposure and analyzed the effects on formation of PLAs via flow cytometry. I hypothesized that acute (4d) exposure to AA will inhibit platelet activation *in vivo* as measured by decreased formation of PLAs. Female mice were exposed to AA (5 ppm; 6h/d, 4d) or HEPA-filtered air (control; 6h/d, 4d). Immediately after final exposure, the mice were euthanized, and their peripheral blood was collected and prepared for flow cytometric analyses for identification and quantification of CD45<sup>+</sup>/CD41<sup>+</sup> PLAs. My results indicate that acute exposure of female mice to AA (5 ppm) yielded no significant change in PLA formation compared with air control (**Figure 20**) [168]. Future studies will include exposing male mice to AA.



**Figure 20.** Effects of acute acetaldehyde (5 ppm) exposure on PLAs in female mice. Flow cytometry analysis was performed in order to identify CD45<sup>+</sup>/CD41<sup>+</sup> PLAs in 100μL female mouse blood following exposure to acetaldehyde (6h/d; n=5,6). Representative flow cytometry dot plots of (A.i.) control (HEPA-filtered air) versus (A.ii.) acetaldehyde-exposed mice are displayed. (B) Summary data displays each individual mouse exposed to either air or acetaldehyde. PLAs are characterized as 20,000 CD45/CD41 double positive events, located in quadrant 2 (Q2). The mice exposed to acetaldehyde showed no difference in PLAs compared with control, p=0.573 via Mann-Whitney U test.

## DISCUSSION

Tobacco consumption is a risk factor that poses a threat to cardiovascular health. Tobacco-derived aldehydes (among other HPHCs) represent a complex mixture of constituents present in MCS. Many of the aldehydes in MCS are also present in e-cig aerosols [51]. Thus, vaping also threatens cardiovascular health. The purpose of this study was to examine the thrombogenic effects of MCS, e-cig aerosols, their humectants (PG and VG), and their aldehyde constituents *in vivo*.

### ***PLA Formation***

I have investigated the level- and duration-dependent effects of exposure to mainstream cigarette smoke (MCS), e-cig aerosol, and aldehydes on PLA formation *in vivo*. Barua and Ambrose state that cigarette smoke exposure (CSE), or MCS, is associated with platelet activation and thrombosis [36]. I hypothesized that MCS exposure causes platelet activation via increased PLA formation *in vivo*. Under the conditions tested, acute exposure to MCS caused a significant increase in PLA formation in C57BL/6 male mice (**Figure 9**). These data serve as a positive control for MCS studies.

Nocella *et al.* 2018 and Carnevale *et al.* 2016 compared the impact of e-cigs with conventional cigarettes in smokers and non-smokers, specifically with endpoints of oxidative stress, vascular function, and platelet function [180, 181]. These studies revealed that exposure to conventional cigarettes induced changes in platelet sCD40L and sP-selectin markers as well as increased collagen-induced platelet aggregation [180]. However, exposure to e-cig did not exert the same effects in these markers. According to

Qasim *et al.* 2019, acute e-cig (with menthol flavorant) exposure leads to several changes in platelet activation markers such as enhanced ADP- and U46619-induced platelet aggregation, enhanced granule secretion, elevated integrin GPIIb-IIIa ( $\alpha_{IIb}\beta_3$ ) activation, enhanced agonist-induced Akt and ERK phosphorylation, and elevated phosphatidylserine expression, though leukocyte activation reportedly did not occur [58]. Likewise, exposure to JUUL (with menthol flavorant), one of the most popular e-cig brands, increases hemostatic activation and thrombus formation, increases platelet sensitivity to ADP- and thrombin-induced aggregation *ex vivo*, and increases platelet granule secretion [59]. I hypothesized that exposure to e-cig aerosols would increase PLA formation *in vivo*. However, under the conditions tested, acute exposure of male mice to e-cig aerosols (classic tobacco flavorant) did not significantly affect PLA formation *in vivo* compared with males exposed to air (**Figure 10**). In the Qasim *et al.* and Montes Ramirez *et al.* publications, both do not observe effects in PLAs as an endpoint of thrombosis. Additionally, both studies used menthol flavorant, whereas this presented study used classic tobacco. These differences in conditions tested may explain why the current study shows that e-cig aerosol is not thrombogenic.

The e-cig humectants PG and VG are GRAS for ingestion, but their inhaled effects on platelet activation is not well-known. In the current study, C57BL/6 male and female mice were exposed to JUUL-like PG:VG (30:70%; v:v), and the effects of PG:VG on PLAs were investigated. Acute exposure of male mice to PG:VG had no significant effect on PLAs compared with control (**Table 4**), yet this exposure significantly increased PLAs in females compared with control (**Table 4**). These data indicate that female mice are more sensitive to PG:VG exposure than males, which is consistent with



our lab's publication showing that the endothelium in female mice is more sensitive to PG:VG exposure than that in males [168].

As previously mentioned, Sithu *et al.* 2010 found that acute (1d, 6h) and sub-chronic (4d, 6h/d as indicated in the literature) exposure to AC (1 ppm) increased PLA formation compared with air control [77]. We believed that exposure to CR (1 ppm) would exert similar effects given the similarities in chemical structure and reactivity. Under the conditions tested in our study, acute exposure to 1 ppm CR resulted in an inhibitory response of PLA formation compared with control (**Table 5**) [81]. A potential hypothesis is that exposure to CR induces NO production. This action causes inhibitory effects of platelet activation, which may help to explain the inhibitory response of PLA formation in our study. More studies are needed to confirm this hypothesis. CR (3ppm) exposure caused an increase in PLA formation (**Table 5**), which may be attributed to activation of the transient receptor potential ankyrin-1 (TRPA1) channel, detailed in **Chapter 4** [81]. Chronic CR exposure had no effect on PLA formation as compared with control (**Table 5**) [81].

To my knowledge, no prior study has explored the relationship between saturated aldehyde exposure and PLA formation as measured by flow cytometry. Under the conditions tested in this study, exposure to FR (1 ppm or 5 ppm), at any duration, exerted no effect on PLA formation in both C57BL/6 males and females compared with control (**Figures 15-19**) [168]. AA exerts inhibitory responses to platelet aggregation in humans and rodents [178, 179]. Under the conditions tested in this study, exposure of C57BL/6 female mice to AA (5 ppm) exerted no significant changes in PLAs compared with control (**Figure 20**) [168]. Our lab has published that e-cig aerosols generate unsaturated

aldehyde at much lower concentrations than saturated aldehydes [65], whereas MCS encompasses high concentrations of all four aldehydes. The presented data suggests that unsaturated aldehydes, not saturated aldehydes, may contribute greatest to MCS-induced PLAs.

### ***PF-4 ELISA***

I have investigated the level- and duration-dependent effects of exposure to PG:VG (30:70%) and AC (1 ppm) on plasma PF-4 levels *in vivo*. PG:VG exposure significantly increased PF-4 levels in female mice (**Figure 12**) but not in the male mice (**Figure 11**). As observed in the PLA data, it appears that female mice are more sensitive to PG:VG exposure than males. With exposure to AC, PF-4 was significantly increased in male mice compared with air control mice (**Figure 13**), in a manner similar to results of Sithu *et al.* [77]. PF-4 was unchanged in female mice exposed to AC compared with air control mice (**Figure 14**).

### ***Limitations of Study***

My results demonstrate that saturated aldehydes exert no effect on PLA formation, whereas I hypothesized that all constituents tested would be pro-thrombotic. However, PLAs are likely not the most sensitive markers of platelet activation and thrombosis. Hence, I explored another sensitive indicator of platelet activation plasma, PF-4. Tail bleeding time assays are also sensitive markers of platelet activation [182, 183] and should be tested in future studies.

## SUMMARY AND CONCLUSIONS

Based on our studies, I conclude that exposure to some HPHCs such as unsaturated aldehydes in tobacco-derived aerosols may play a significant role in platelet activation and, thereby, increase both PLAs and PF-4. More research is required to explore pro-thrombotic mechanisms and how HPHCs influence those mechanisms. A mechanism explored in this study is how TRPA1 potentially mediates pro-thrombotic effects of HPHCs (**Chapter IV**).

## CHAPTER IV

### EFFECT OF INHALED ALDEHYDES ON PLATELET ACTIVATION *IN VIVO*:

#### THE ROLE OF TRANSIENT RECEPTOR POTENTIAL ANKYRIN-1

##### INTRODUCTION

Tobacco-derived HPHCs can induce CVD, in part by platelet activation. However, the specific mechanisms are unclear. A mechanism I explored for cigarette smoke-induced platelet activation, thrombosis and subsequent CVD is the role of transient receptor potential ankyrin-1 (TRPA1). TRPA1 belongs to a large class of cation channels widely expressed throughout the sensory nervous system [98], and it is present throughout the cardiopulmonary system. TRPA1 is a pain signaling/sensory cation channel that can be activated by environmental irritants such as  $\alpha,\beta$ -unsaturated aldehydes AC [100, 111, 184, 185] and CR [80, 81], as well as flavorant aldehydes like cinnamaldehyde [94, 103, 186]. As described in **Chapter I**, TRPA1 channel activation in endothelial cells allows calcium influx into cells, which leads to NO release that is produced from L-arginine via endothelial NO synthase (eNOS) [187]. In sensory neurons, calcium entry leads to vesicle:cell membrane fusion and release of vasoactive peptides calcitonin gene-related peptide (CGRP) and substance P (SubP) [107, 108]. These peptides increase vascular permeability, increase blood flow, and increase leukocyte binding in the vasculature via a process called neurogenic inflammation [98, 100, 185, 188, 189]. Unsaturated aldehydes such as AC, bind to the N-terminal cysteines, triggering TRPA1 to allow calcium entry, leading pro-inflammatory signaling, inflammation, and cell death [190]. Platelet activation is a pro-inflammatory effect [183,

191-193]. Thus, activation of the TRPA1 channel may elicit effect on platelets via an indirect mechanism.

Our previous studies show that AC and CR activates TRPA1 and leads to TRPA1-dependent vascular toxicity [80, 81, 111]. However, the connection between TRPA1 activation and platelet activation is unknown. We are the first research lab to investigate whether TRPA1 activation contributes to the potential thrombogenic effects associated with exposure to tobacco-derived HPHCs. Here, I have evaluated the role of TRPA1 in tobacco-derived HPHC-induced platelet activation and thrombosis in WT and TRPA1-null mice via PLA and plasma PF-4 assays.

## MATERIALS AND METHODS

### *Materials*

Reagent-grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

### *Methods*

Mice: As described in Lynch *et al.* 2020 [81], male and female C57BL/6J (wild type, WT) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Male TRPA1-null mice (on a C57BL/6J background) were from a breeding colony at the University of Louisville [111]. 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. Before and during exposures, mice were housed 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).

E-cig Aerosol Exposure System: As described in Jin *et al.* [168], a software-controlled (FlexiWare) cigarette-smoking robot (CSR; SCI-REQ; Montreal, Canada) system was used in the mechanical generation of aerosols from PG:VG mixtures. To control the generation of volatile organic compounds (VOCs) in e-cig aerosols, we used a defined e-cig platform. PG:VG mixture (30:70 ratio, vol/vol) was loaded into a refillable, clear tank atomizer with a fixed coil resistance (Mistic Bridge; ~3.0 ohm; purchased online) coupled with a rechargeable bluPLUS+ (3.7 V) battery (power out-put of ~8W). The

atomizer tank was weighed before and after use to quantify solution consumption (g/puff). A 9-min session was composed of 18 puffs (4 s/puff, 91.1 mL/puff, 2 puffs/min). For the exposures, 20 sessions were evenly spaced over a 6h exposure per day for 4 consecutive days. Total suspended particulate (TSP) matter was monitored in real time with an inline infrared Microdust Pro 880 nm (Casella) positioned upstream of the exposure chamber (5 L, SCI-REQ). All whole-body exposures were done between 7:00AM and 2:00PM in the absence of food or water. Immediately following the final exposure, mice were euthanized by sodium pentobarbital ( $\approx 150$  mg/kg, i.p.) and ventral thoracotomy and exsanguination with cardiac puncture for peripheral blood collection in EDTA-coated syringes.

*Acrolein and Crotonaldehyde Exposures:* To parallel our previous study of chronic (12 weeks, 1 ppm) acrolein (AC) exposure in mice [167] naïve mice were exposed to HEPA- and charcoal-filtered air, AC at 1 ppm, or crotonaldehyde (CR) for 4 consecutive days (1 or 3 ppm, 6h/d) or 12 weeks (1 ppm; 6h/d, 5 d/week) using a custom exposure system and a certified permeation tube (Kin-Tek; LaMarque, TX). All whole-body exposures were done between 7:00AM and 2:00PM in the absence of food or water. Immediately following the final exposure, mice were euthanized by sodium pentobarbital ( $\approx 150$  mg/kg, i.p.) and ventral thoracotomy and exsanguination with cardiac puncture for peripheral blood collection in EDTA-coated syringes.

*Platelet-Leukocyte Aggregates (PLAs):* After the final exposure, the mice were immediately euthanized and peripheral blood collected and used to detect PLAs. PLAs were identified by flow cytometry and quantified as events double positive for CD41 (platelets) and CD45 (leukocytes) as previously described in Conklin *et al.* [167] with

slight modifications. Briefly, aliquots of whole blood were diluted (1:4) with HEPES-Tyrode solution before fixation [paraformaldehyde, Fc 1.6%, room temperature (RT)]. Red blood cells were lysed (MilliQ water), and the sample was centrifuged (400g, 5 min, RT). The sample pellet was incubated with 1% Fc Block (5 $\mu$ L; 10 min) before staining for 30 min with FITC-labeled anti-CD41 and APC-labeled anti-CD45 or isotype-matched negative controls (FITC-IgG1; APC-IgG2bk). Stained cells were washed with HEPES-Tyrode solution containing 1% BSA, centrifuged at 400g for 5 min, and resuspended in HEPES-Tyrode solution (250 $\mu$ L). A BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA) was used to analyze stained cells; a minimum of 20,000 events was collected for each sample.

*Platelet Factor 4 (PF-4) Assay:* As described by Sithu *et al.* [77], plasma PF-4 levels were assayed by a sandwich ELISA using DeoSet Mouse PF-4/CXCL4 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, 96-well ELISA plates were coated with a rat anti-mouse PF-4 capture antibody (2 $\mu$ g/mL in PBS) for 16h-overnight at room temperature. Wells were washed free of the unbound antibody and blocked with 1% BSA for 1h at room temperature. Plasma samples or the PF-4 standards were incubated in the coated wells for 2h at room temperature. Wells were washed three times and then incubated with a biotinylated goat anti-mouse PF-4 antibody (100ng/ ml) for 2h at room temperature. Wells were washed again and then incubated with streptavidin conjugated to horseradish-peroxidase for 20 min at room temperature. The washing step was repeated and the substrate, tetramethylbenzidine (TMB) was added and the reaction was incubated for 15 min. The reaction was stopped



with 1N H<sub>2</sub>SO<sub>4</sub> and the color developed was measured using a microplate reader at 450 nm.

*Statistical Analysis:* Data are presented as means  $\pm$  standard error (SE). For statistical comparison between groups, the Mann-Whitney U test or the Two-Way ANOVA with Tukey post hoc test was used where appropriate. Significance was accepted where  $p < 0.05$ .

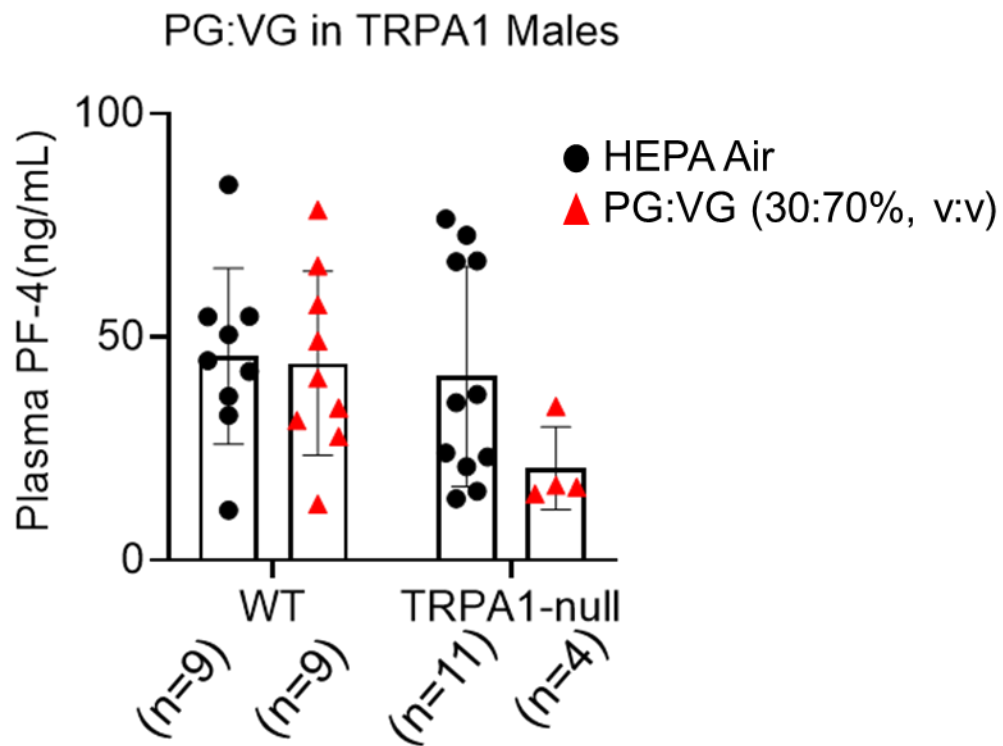
## RESULTS

**A. Effects of PG:VG exposure on PLAs and plasma PF-4 levels in TRPA1 WT and null male and female mice.** In WT animals exposed to PG:VG (30:70) (**Tables 4 and 6**), our results indicate that PG:VG exposure in female mice yields a significant increase in % PLAs compared with air-exposed female mice. In males there were no differences between PG:VG exposure and air exposure. I hypothesized that exposure to PG:VG aerosols in WT mice would induce a TRPA1-dependent PLA increase. In **Table 6**, we exposed TRPA1-null male and female mice to PG:VG (30:70). Our results indicate a significant decrease in % PLAs in female TRPA1-null mice exposed to PG:VG compared with WT. Thus, the effects of PG:VG in female mice are TRPA1-dependent. No observed difference of PLAs in males occurred, regardless of their TRPA1 genotype. In **Figure 21**, there were no differences in PF-4 levels in WT or TRPA1-null male mice compared with control. Interestingly, in **Figure 22**, PF-4 levels in PG:VG exposed WT females mice significantly increased, and PF-4 appeared to return to baseline in the TRPA1-null females. This is indicative of a TRPA1-dependent effect of PG:VG in female mice with regards to PF-4 levels.

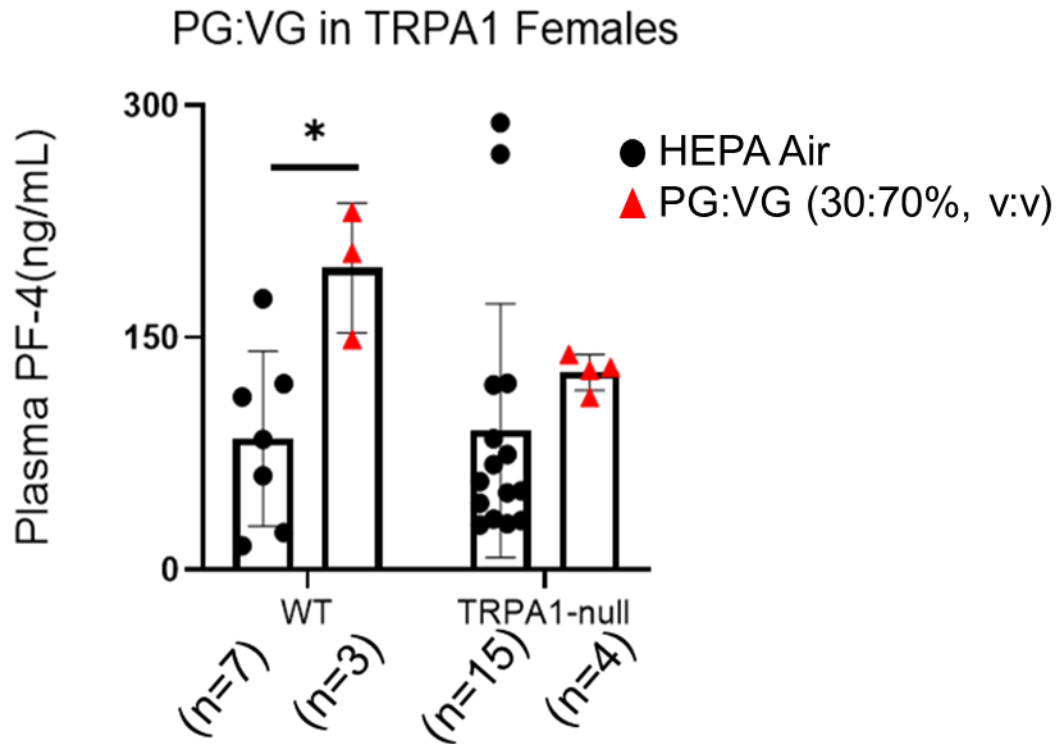
**Table 6.** PLA (%) changes in TRPA1 WT (C57BL/6 background) and TRPA1-null male and female mice acutely exposed to air or PG:VG. Flow cytometry analyses were performed in order to identify CD45<sup>+</sup>/CD41<sup>+</sup> PLAs in 100μL male mouse blood following exposure to air, PG:VG (30:70), or AC (1 ppm). PLAs are characterized as 20,000 CD45/CD41 double positive events, located in quadrant 2 (Q2).

	<b>Exposure (6h/d; 4d)</b>			
	<b>Air Control</b>		<b>PG:VG (30:70)</b>	
	<b>WT</b>	<b>TRPA1-null</b>	<b>WT</b>	<b>TRPA1-null</b>
<b>Male PLAs (% leukocytes)</b>	6 ± 1	2 ± 1	6 ± 1	4 ± 2
<b>Female PLAs (% leukocytes)</b>	6 ± 3	4 ± 0	13 ± 2*	2 ± 0*

Values are means ± SE; n = 3-25 mice per group. Abbr.: PG; propylene glycol; PLAs, platelet-leukocyte aggregates; TRPA1, transient receptor potential ankyrin-1; VG, vegetable glycerin.



**Figure 21.** Effects of PG:VG (30:70) exposure on PF-4 levels in plasma of TRPA1 WT and null male mice. PF-4 from mouse plasma was measured via sandwich ELISA. Across all groups, the data result in insignificance via Two-Way ANOVA with Tukey post hoc test.



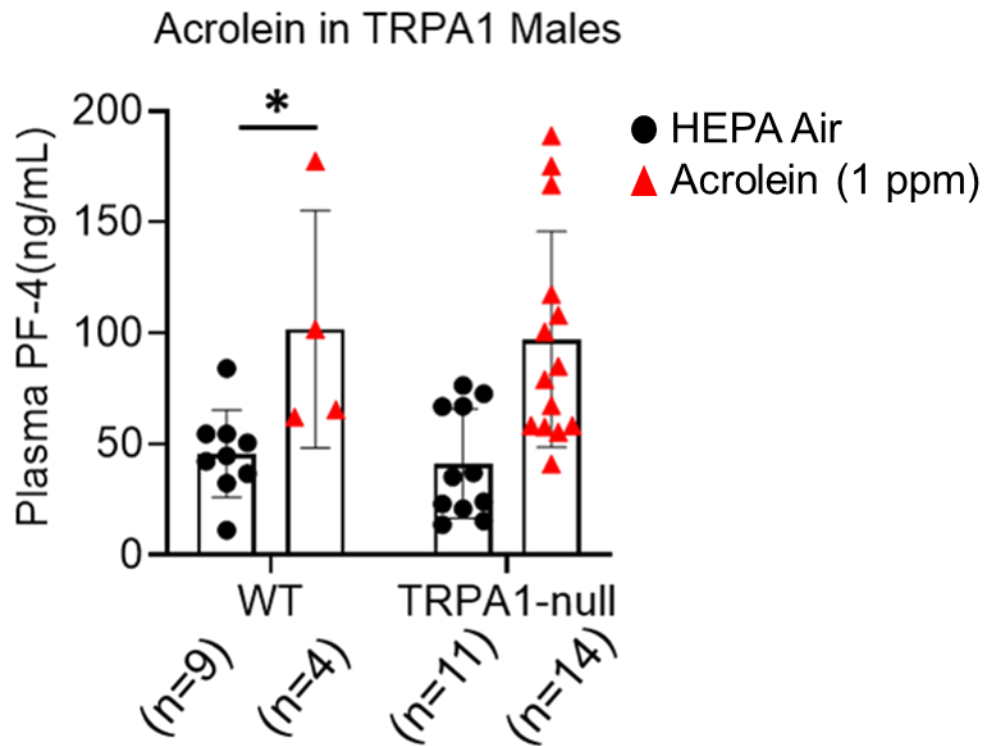
**Figure 22.** Effects of PG:VG (30:70) exposure on PF-4 levels in plasma of TRPA1 WT and null female mice. PF-4 from mouse plasma was measured via sandwich ELISA. WT mice exposed to PG:VG have a significant difference in PF-4 levels compared with control, via Mann-Whitney U test. This effect is TRPA1-dependent via Two-Way ANOVA with Tukey post hoc test.

**B. Effects of 1 ppm AC exposure on PLAs and plasma PF-4 levels in TRPA1 WT and null male and female mice.** Sithu *et al.* acutely exposed WT male mice to 1 ppm AC and saw a significant increase in % PLAs compared with air control [77]. Under similar conditions in TRPA1 WT and null animals exposed to 1 ppm AC (**Table 7**), my results indicate that AC exposure did not significantly affect PLAs in these animals. Sithu *et al.* also found that acute exposure to AC results in a significant increase in PF-4 levels in males [77]. Interestingly, in **Figure 23**, PF-4 levels are elevated in AC exposed WT and TRPA1-null males, though insignificant. PF-4 levels are unchanged in WT and TRPA1-null females exposed to AC (**Figure 24**).

**Table 7.** PLA (%) changes in TRPA1 WT (C57BL/6 background) and TRPA1-null male and female mice acutely exposed to air or acrolein (AC). Flow cytometry analyses were performed in order to identify CD45<sup>+</sup>/CD41<sup>+</sup> PLAs in 100µL male and female mice blood following exposure to air, AC (1 ppm). PLAs are characterized as 20,000 CD45/CD41 double positive events, located in quadrant 2 (Q2).

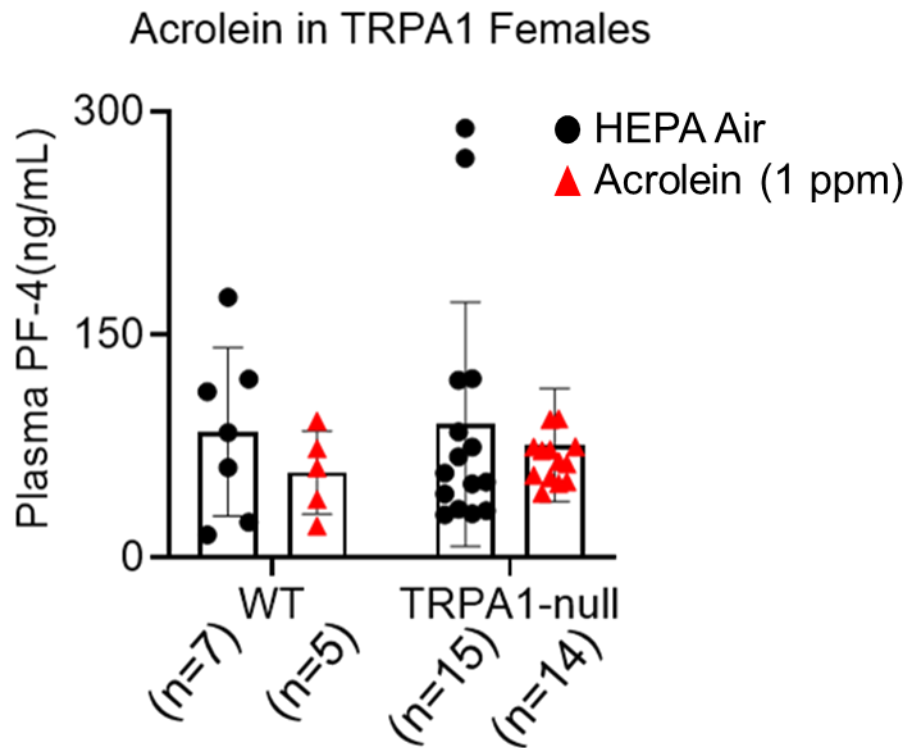
	<b>Exposure (6h/d; 4d)</b>			
	<b>Air Control</b>		<b>AC (1 ppm)</b>	
	<b>WT</b>	<b>TRPA1-null</b>	<b>WT</b>	<b>TRPA1-null</b>
<b>Male PLAs (% leukocytes)</b>	6 ± 1	2 ± 1	7 ± 1	5 ± 1
<b>Female PLAs (% leukocytes)</b>	6 ± 3	4 ± 0	8 ± 2	4 ± 0

Values are means ± SE; n = 3-25 mice per group. Abbr.: AC; acrolein; PLAs, platelet-leukocyte aggregates; TRPA1, transient receptor potential ankyrin-1.



**Figure 23.** Effects of acrolein (1 ppm) exposure on PF-4 levels in plasma of TRPA1 male mice. PF-4 from mouse plasma was measured via sandwich ELISA. Exposure to acrolein in both WT and TRPA1-null mice show significant increases in PF-4 levels compared with WT air control, via Two-Way ANOVA with Tukey post hoc test.





**Figure 24.** Effects of acrolein (1 ppm) exposure on PF-4 levels in plasma of TRPA1 female mice. PF-4 from mouse plasma was measured via sandwich ELISA. Across all AC exposures, no significant differences were detected, via Two-Way ANOVA with Tukey post hoc test.

**C. Effects of CR exposure on PLAs in TRPA1 male mice.** In WT male mice acutely (4d) exposed to 1 ppm or 3 ppm CR (**Table 5**), our results indicate significant changes. Exposure to 1 ppm CR significantly decreased % PLAs compared with control [81]. Therefore, I hypothesized that the effects of CR are TRPA1-dependent. In **Table 8**, we exposed TRPA1-null male mice to 1 ppm CR. Our results indicate that % PLAs in the TRPA1-null mice return to baseline as observed in air-exposed WT male mice. I conclude the effect of 1 ppm CR is TRPA1-dependent [81].

**Table 8.** PLA (%) changes in TRPA1 WT (C57BL/6 background) and TRPA1-null male mice acutely (4d) exposed to air or crotonaldehyde (CR). Flow cytometry analyses were performed in order to identify CD45<sup>+</sup>/CD41<sup>+</sup> PLAs in 100µL male mouse blood following exposure to CR (1 or 3 ppm). PLAs are characterized as 20,000 CD45/CD41 double positive events, located in quadrant 2 (Q2).

	Exposure (6h/d; 4d)				Reference
	Air Control		CR (1 ppm)		[81]
	WT	TRPA1-null	WT	TRPA1-null	
<b>PLAs (% leukocytes)</b>	6 ± 1	7 ± 3	3 ± 0*	6 ± 1	

Values are means ± SE; n = 9-15 mice per group. Abbr.: CR: crotonaldehyde; PLAs, platelet-leukocyte aggregates; p<0.05 compared with air control based on Bonferroni's post-test.

## DISCUSSION

### *PLA Formation*

The present study investigated the effects of acute exposures to PG:VG aerosols and unsaturated aldehyde gases on PLA formation in TRPA1-WT and TRPA1-null mice *in vivo*. Previously shown, acute exposure to MCS causes a significant increase in PLA formation in C57BL/6 male mice (**Figure 9**). Additionally, it was concluded that the effects of MCS on PLAs were due to the presence of the unsaturated aldehydes (AC and CR) and not the saturated aldehydes (FR and AA). However, the mechanism how AC and/or CR elicit increased PLAs was not determined. It is known that  $\alpha,\beta$ -unsaturated aldehyde AC and TRPA1 mediate MCS-induced neurogenic inflammation [110]. Therefore, it was hypothesized that TRPA1 mediates  $\alpha,\beta$ -unsaturated aldehyde-induced platelet activation via increased PLAs and increased PF-4 levels.

Our lab has published that acute exposure of TRPA1-WT males to PG:VG (30:70) does not induce a significant change in PLA formation compared with air control [168]. In TRPA1-null males exposed to PG:VG, we also did not observe any effects on PLAs (**Table 6**). Additionally, % PLAs were significantly increased in WT females exposed to PG:VG (**Table 6**). This effect of PLAs was abolished in TRPA1-null females exposed to PG:VG, indicative of a TRPA1-dependent effect of PG:VG in female mice (**Table 6**).

As stated previously, Sithu *et al.* found that acute (1d, 6h) and sub-chronic (4d, 6h/d) exposure to AC (1 ppm) increased PLA formation in WT male mice compared with air control [77]. Under similar testing conditions, our results were negative in WT males

and females exposed to AC (**Table 7**). Sithu *et al.* measures PLAs as CD11b<sup>+</sup>/CD41<sup>+</sup> (monocytes/platelets) events [77], whereas the current data measures PLAs as CD45<sup>+</sup>/CD41<sup>+</sup> (leukocytes/platelets) events. Monocytes are the largest type of leukocyte and represent approximately 2-8% of total leukocytes. The data by Sithu *et al.* could give a mechanistic insight into how acrolein specifically targets and activates monocytes rather than all leukocytes combined (e.g. neutrophils, lymphocytes, eosinophils, and basophils). Therefore, increased platelet-monocyte aggregate formation may be the effect of AC exposure *in vivo*. With regards to female mice, we have published that the TRPA1 channel protects against high level AC exposure in a sex-dependent manner in which the WT females are more protected [111]. I expected to see PLAs increased in TRPA1-null female mice. However, I found no changes in PLAs of TRPA1-null female mice exposed to AC compared with control (**Table 7**).

We believed that exposure to CR (1 ppm) would exert similar effects as AC given the similarities in chemical structure and reactivity. Under the conditions tested in our study, acute exposure of WT male mice to 1 ppm CR decreased PLA formation relative to control (**Table 8**) [81]. When testing the effects of 1 ppm CR in TRPA1-null male mice, PLAs were unaffected relative to control (**Table 8**), an indication of TRPA1-dependent effects of CR on platelets. As previously mentioned in **Chapter III**, a potential hypothesis is that exposure to CR may induce NO production in the endothelium [80]. It is known that NO inhibits platelet activation [121, 194, 195], which may help to explain the inhibitory response of PLA formation. It is known that TRP channels including TRPA1 and TRPV1 mediate nitric oxide (NO)-induced nociception in mice [196]. NO activates TRPA1, induces calcium influx in TRPA1-expressing cells, and

activates the dorsal root ganglia (DRG) [196, 197]. In my PLA data, it appears that 1 ppm CR suppresses PLA formation in a TRPA1-dependent manner. More studies are needed to determine if the PLA suppression from CR exposure is also due NO production by the endothelium.

### ***PF-4 ELISA***

To my knowledge, no study shows a relationship between the TRPA1 channel and PF-4. I have investigated the effects of PG:VG (30:70%, v:v) and the  $\alpha,\beta$ -unsaturated aldehyde AC (1 ppm) on plasma PF-4 of TRPA1 WT and TRPA1-null male and female mice via sandwich ELISA *in vivo*. PG:VG exposure had no significant effect on PF-4 levels in WT and null male mice compared with respective air control mice (**Figure 21**). Results from female mice exposed to PG:VG indicate an increase in PF-4 levels in WT compared with control (**Figure 22**). This effect proved to be TRPA1-dependent in that PF-4 levels were unaffected in TRPA1-null females exposed to PG:VG. Together, these data indicate sex- and TRPA1-dependent effects of PG:VG on mouse PF-4 *in vivo*. This sex-dependent effect of PG:VG exposure on the endothelium has also been shown [168].

As previously mentioned, Sithu *et al.* show that acute AC (1 ppm) exposure significantly increases plasma PF-4 levels in WT male mice. It was not determined if the effect was TRPA1-dependent. In our model, AC exposure in WT males increases PF-4 (**Figure 23**). In TRPA1-nulls exposed to AC, PF-4 is also elevated which indicates that the effects of AC are not TRPA1-dependent in male mice *in vivo*. Our previous data show that TRPA1 protects against high-level acrolein exposure in a sex-dependent manner [111]. TRPA1 protects female mice from irritant exposures like acrolein [111]. In **Figure 24**, WT and null female mice exposed to 1 ppm AC had no difference of PF-4 levels

compared with control. Together, these data indicate TRPA1-dependent and sex-dependent effects of AC on mouse PF-4, though more studies are needed understand the mechanism between activation of TRPA1 and degranulation of PF-4.

## **SUMMARY AND CONCLUSIONS**

Based on our studies, I conclude that TRPA1 may indirectly mediate the effects observed due to exposure of platelets to  $\alpha,\beta$ -unsaturated aldehydes in tobacco-derived aerosols. These aldehydes act through the TRPA1 channel to contribute to overall MCS-induced platelet activation. In addition, the pro-thrombotic effects associated with PG:VG and AC exposures are sex-dependent.

## CHAPTER V

### SUMMARY AND CONCLUSIONS

#### MAJOR FINDINGS IN THIS DISSERTATION

Tobacco-derived HPHCs such as flavorants and aldehydes may induce platelet activation *ex vivo* or *in vivo*. As stated in **Chapter I**, the purpose of this dissertation is to investigate the effects of HPHCs on pro-thrombotic outcomes and to evaluate if the observed effects were mediated by the TRPA1 channel. I developed three **Specific Aims** to test if exposures to HPHCs either alone or as present in MCS or e-cig-derived aerosols elicited pro-thrombotic responses in platelets.

The goal of **Aim 1** was to determine the direct effects of individual HPHCs, specifically of 15 common e-cig flavorant additives, on biphasic platelet aggregation *ex vivo* using isolated human PRP. I recorded the responses of platelets (incubated with a flavorant) due to ADP stimulation and measured total aggregation, phase 1 aggregation, and phase 2 aggregation through 5 mins. Analyzing these biphasic responses of ADP-induced platelet aggregation allows for a mechanistic approach for evaluating how parent flavorants sensitize platelets via potentiating, inhibiting, or not affecting the ADP-specific P2Y<sub>1</sub> and P2Y<sub>12</sub> pathways of platelet activation. Phase 1 strictly depends on exogenous ADP addition. Phase 2 occurs from intrinsic release and activation of platelet  $\alpha$ -granules and dense granules and amplifies aggregation. Of the 15 parent flavorants tested, only eugenol elicited effects, inhibiting the Phase 2 response and thus inhibiting total aggregation. These data indicate flavorants, except eugenol, neither activate or inhibit ADP-induced platelet aggregation. Consistently, eugenol is known to be a strong anti-clotting compound.



The goal of **Aim 2** was to quantify the level- and time-dependence of HPHCs, specifically aldehydes, exposure on pro-thrombotic alterations *in vivo*. C57BL/6 male and female mice underwent acute (4d) and chronic (CR; 12wk) whole-body exposures to aldehydes, and I evaluated the effects of HEPA-filtered air (control), MCS (12 cigs/d), e-cig aerosols, PG:VG (30:70), and saturated (FR and AA) and unsaturated aldehydes (AC and CR) on PLA formation and plasma PF-4 levels. Under the conditions tested, MCS and unsaturated aldehyde exposures induced pro-thrombotic outcomes in male mice as assessed via flow cytometry analyses. MCS, 1 ppm AC, and 3 ppm CR significantly increased PLAs in males compared with control. Saturated aldehyde exposure caused no significant changes on PLAs in males or females. These data indicate that MCS-induce PLA formation is due to the presence of the unsaturated aldehydes AC and CR.

PF-4 is an index and a sensitive marker of platelet activation and thrombosis [198]. Post-exposure to HEPA-filtered air, PG:VG and AC, I completed ELISAs to evaluate PF-4 levels in plasma. There were significant changes in PF-4 levels due to PG:VG exposure in WT females compared with control. After exposure to 1 ppm AC, male PF-4 levels increased in WT mice compared with control. These data indicate that PG:VG exposure in females and AC exposure in males activate platelets and stimulates degranulation of  $\alpha$ -granules releasing PF-4 into the blood.

The goal of **Aim 3** was to test the role of the TRPA1 channel in aldehyde-induced platelet activation. PG:VG exposure in WT female mice increased PLA formation and increased PF-4 levels. The effects of PG:VG on PLAs and PF-4 returned to baseline in the TRPA1-null females compared with control. This is indicative of a TRPA1-dependent

effect of PG:VG exposure in females. With no changes in PG:VG exposed TRPA1 WT and null male mice, these data also suggest a sex-dependent effect of PG:VG on platelets.

AC exposure in WT male mice increased PLAs [77] and PF-4 levels. This effect of AC persisted in TRPA1-null males. Therefore, this effect is not TRPA1-dependent, but these data indicate AC as a potent toxicant.

## **STRENGTHS**

There are strengths in my human and murine exposure studies. With regards to the flavorant data in isolated human platelets in **Chapter II**, our lab is the first to the investigate effects of common parent flavorants on ADP-induced biphasic human platelet aggregation. Because our study used human platelets instead of animal platelets, we are able to more effectively model the effects of flavorants on human health. Therefore, making inferences for overall effects on humans is easier to construct. This study also benefits from studying the pure compounds rather than complex flavor profiles that are diverse and can complicate experimentation. Another strength of this study involves justifying the appropriate test concentration was used that mimics real-world flavorant consumption during e-cig use. The levels of flavorants in the blood are not reported. Therefore, I estimated potential blood levels of flavorants based on reported concentrations of flavorants in the e-liquids. These estimations are based on convenient assumptions, e.g., 100% efficiency in both transfer from e-liquid to aerosol and from aerosol to the blood. After performing calculations, I was able to determine appropriate test concentrations of flavorants to use in the study. Lastly, the final strength of this study is determining eugenol as a strong positive control flavorant. Many studies have demonstrated the anti-thrombotic effects of clove oil and its major constituent eugenol.

Our study also shows the strong inhibitory effect of eugenol on platelets directly. Thus, we were able to conclude that eugenol is a potent positive control for anti-clotting studies.

With regards to my whole-body murine studies in **Chapter III**, our lab has completed urinary metabolite studies showing the levels of FR, AA, AC and CR metabolites following exposure to MCS. These data helped in validating the concentration of aldehydes to test in our whole-body exposure studies.

We are the first lab to investigate the role of TRPA1 in mediating the effects of aldehydes in tobacco aerosols, specifically the  $\alpha,\beta$ -unsaturated aldehydes AC and CR, on pro-thrombotic outcomes. We show that the effects of AC and CR on PLAs and PF-4 are TRPA1-dependent via flow cytometry analysis and via ELISA. Finally, we are the only lab to show that there are sex-dependent differences regarding the TRPA1 channel, in that TRPA1 protects female mice against high level AC exposure. In addition, the present study shows that there are sex-dependent differences due to PG:VG exposure, in that females are more sensitive than males to PG:VG exposure.

## **LIMITATIONS**

I have identified important limitations within my human and murine studies. With regards to my flavorant data in isolated human platelets (**Chapter II**), it is important to know that flavorants are heated during e-cig use. The parent flavorants undergo thermal degradation that can lead to daughter compounds that could potentially be more toxic than the parents, such as flavor acetals [91]. Additionally, we know e-cigs contain nicotine, an addictive substance. Studies involving flavor acetals and nicotine are important considerations to mimic real-world e-cig use. I did not test for the effects of

nicotine alone or in combination with flavors on platelets. It has been shown that in isolated PRP exposed to nicotine (10 mM) from non-smokers, ADP- and 5-hydroxytryptamine (5-HT, serotonin)-induced platelet aggregation was potentiated [199, 200]. Nicotine's effect on collagen-, ristocetin-, adrenaline-, and arachidonic acid-induced platelet aggregation was inhibited. This is indicative of receptor-dependent effects of nicotine.

With regards to my murine data in **Chapters III and IV**, it is important to know that mice are obligate nose breathers and are “involuntary smokers.” These animals have a reflex in which they decrease their breathing rate and depth in order to reduce the amount of toxins, like acrolein, they are inhaling [111]. This reflex is termed “respiratory braking” [201]. We also know that a mouse nose is very sensitive to aldehyde exposure that can lead to excessive swelling and blockage in the nose, and subsequently, to death due to congestion and hypoxia [185].

Under the conditions tested, FA and AA, as well as 1 ppm CR do not induce platelet activation. This could be because these levels of exposure may be insufficient to elicit effects in platelets. FA concentrations in MCS and e-cig aerosols can be >6 ppm [64, 202, 203]. Blood AA concentrations from MCS exposure range  $\sim 16 \pm 5 \mu\text{M}$  [204]. We don't usually report solution concentrations in ppm, more typically mg/l or molar. The concentration of CR in MCS ranges from 1-53 ppm [205]. The concentrations used in this study are within range of MCS exposure, but it is possible that higher concentrations of the compounds induce platelet activation. Although some of our studies only had male mice, several of our studies had sex-dependent comparisons.

Additional studies will provide information on sex differences, which in our hands were intriguing.

Lastly, it is important to address variability in the data as a source of limitations. In the *in vivo* study, blood is drawn in EDTA-coated 22G syringes and transferred into tubes containing EDTA to prevent from spontaneous clotting due to blood draw. Male mice have larger hearts than female mice, thus drawing peripheral blood from female hearts is more challenging. Though the syringes are coated in EDTA, it is possible that drawing the blood too fast may affect platelet function alone. When preparing whole blood for flow cytometry analyses, the blood is constantly handled in ways that may inadvertently activate platelets such as vortexing the tubes, centrifuging the tubes, adding volumes of buffer, etc... Together, each of these actions in blood draw and benchwork handling may influence the variability of the data, especially in the control blood samples.

## **FUTURE DIRECTIONS**

Future studies are important and necessary in order to completely understand the scope of toxicities associated with exposure to tobacco smoke and HPHCs. First, tobacco products are heated instruments. Thus, flavorants and PG:VG (solvents in e-cig liquids) are also heated and can degrade to daughter compounds, e.g., aldehydes, that are potentially more toxic. In the human studies, future studies are to include testing the effects of heated flavorants on human biphasic platelet aggregation. Additionally, future studies are to include testing the direct effects of individual aldehydes on isolated platelets. Selley *et al.* reports high level AC exposure (50-5,000  $\mu\text{M}$ ) in isolated human PRP had no significant effect on platelet aggregation response to ADP, epinephrine,

collagen, or ionophore A23187 [206]. However, these levels of AC exceed the levels a smoker would be exposed to, and therefore, lower concentrations should be tested.

Zoucas and Bengmark report that ethanol and its aldehyde metabolite, AA, inhibits ADP- and collagen-induced platelet aggregation in rat PRP [179]. The direct effects of CR and FA on isolated PRP has not been investigated.

Second, tobacco aerosols are composed of a complex mixture of HPHCs, including nicotine. When a person smokes or vapes, they are being exposed to nicotine. Moreover, the toxicity associated with nicotine exposure on pro-thrombotic has not been thoroughly investigated. Future studies should interrogate the role of nicotine in tobacco smoke-induced thrombosis.

Third, as previously mentioned, studies involving thrombotic outcomes include a variety of platelet function assays tests. Future studies should incorporate other platelet assays and evaluate as many thrombotic endpoints as possible. For example, bleeding time is a procedure done to assess platelet function and coagulation *in vivo*. Tail bleeding time assays have been routinely performed in animal models to resemble human bleeding disorders [207, 208]. This quantitative measurement of hemostasis in mice gives insight into the rapid responses of platelet activation. These assays involve transecting the tip of the tail to initiate bleeding, submerging the tail into a saline solution, and documenting the time it takes for the tail to cease bleeding or hemorrhaging. The time is a representation of slow or rapid clotting due to platelet activation. Qasim *et al.* demonstrated the post-exposure to e-cig aerosols, tail bleeding and occlusion time were shortened in C57BL/6 male mice, indicating a rapid clotting response [58]. Sithu *et al.* also demonstrated that acute exposure (4d, 6h/d) to 1 ppm AC shortens tail bleeding time [77].

Other endpoints to evaluate are platelet adhesion, platelet granule release such as serotonin-release, and platelet aggregation.

Lastly, TRP channels are divided into seven subfamilies: TRPC, TRPV, TRPM, TRPN, TRPA1, TRPP, TRML [209]. Each may be activated by different stimuli than others which can lead to different physiological outcomes. It is possible that platelet activation is mediated by a different TRP channel. For example TRPM7 is essential in platelet function in mice [210], and it is involved with regulating thrombus growth and hemostasis in mice [211]. TRPV1 has been detected in human platelets but not in mouse platelets [212]. In another example, platelets express TRPC [213, 214]. TRPC channels can regulate nicotine-dependent behavior [215] and may regulate MCS-induced cardiopulmonary toxicity [216]. In this dissertation, however, given that MCS and aldehyde constituents trigger TRPA1 specifically, the focus was to investigate and understand how aldehydes in tobacco-derived aerosols trigger platelet activation and if TRPA1 mediates the effects indirectly. Future studies should include investigating tobacco-derived aldehyde-induced platelet activation and thrombosis and the potential role of other TRP channels.

## CONCLUSIONS

Overall, this study demonstrates the pro-thrombotic effects of tobacco-derived aerosols. I was able to describe how flavorants have limited to no direct toxicity on platelets. I was also able to describe how aldehydes in tobacco aerosols may induce pro-thrombotic effects *in vivo*. In conclusion, the data presented in this dissertation indicate that short-term exposures of mice to MCS and unsaturated aldehydes (AC and CR) induce pro-thrombotic outcomes. My hypothesis is supported in that HPHCs, specifically

aldehydes, have a significant role in tobacco smoke-induced platelet activation and thrombosis, and that TRPA1 may mediate some of these outcomes. These data can be used by the FDA to better regulate the presence of HPHCs to decrease morbidity and mortality rates due to smoking.



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## APPENDICES

### ABBREVIATIONS

AA	Acetaldehyde
AC	Acrolein
ADP	Adenosine diphosphate
AHA	American Heart Association
CDC	Centers for Disease Control and Prevention
CGRP	Calcitonin gene-related peptide
CR	Crotonaldehyde
CSR	Cigarette-smoking robot
CVD	Cardiovascular disease
E-cig	Electronic cigarette
EDTA	Ethylenediaminetetraacetic acid
ENDS	Electronic Nicotine Delivery Systems
eNOS	Endothelial nitric oxide synthase
FA	Formaldehyde
FDA	U.S. Food and Drug Administration
GRAS	Generally regarded as safe
HEPA	High-efficiency particulate air
HPHCs	Harmful or potentially harmful constituents
ISO	International Standard of Organization
MCS	Mainstream cigarette smoke
NO	Nitric oxide
NTP	National Toxicology Program
OHSA	Occupational Safety and Health Administration
P1	Phase 1 platelet aggregation

P2	Phase 2 platelet aggregation
PAF	Platelet activating factor
PF-4	Platelet factor 4
PG	Propylene glycol
PLAs	Platelet-leukocyte aggregates
PMA	Platelet-mononuclear cell aggregates
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
ROS	Reactive oxygen species
RT	Room temperature
SubP	Substance P
TMB	Tetramethylbenzidine
TRPA1	Transient receptor potential ankyrin-1
TSP	Total suspended particulate
VG	Vegetable glycerin
WT	Wild Type
5-HT	5-hydroxytryptamine (serotonin)

## CURRICULUM VITAE

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### **EDUCATION**

**2020-present** University of Louisville, Louisville, KY  
Ph.D. Candidate Pharmacology and Toxicology  
**2016-2020** University of Louisville, Louisville, KY  
M.S. Pharmacology and Toxicology  
**2014-2016** Nazareth College of Rochester, Rochester, NY  
B.S. Cellular Toxicology  
B.S. Biology  
Minor in Chemistry  
**2011-2013** Mercer University, Macon, GA

### **HONORS**

**2022-** Honoree at UofL School of Medicine graduation celebration for Underrepresented Minority MDs, PhDs, and Trainees  
**2021-** Board of Directors (West Jefferson County Community Task Force)  
**2021-** 1<sup>st</sup> place award for best oral elevator pitch at the Diabetes and Obesity Center (DOC) Trainee Retreat  
**2020-** 1<sup>st</sup> place award for best Ph.D. poster presentation at the Ohio Valley Society of Toxicology (OVSOT) virtual meeting  
**2020-** 1<sup>st</sup> place award for best graphical abstract presentation at the Ohio Valley Society of Toxicology (OVSOT) virtual meeting  
**2019-** Awarded NIEHS T32 Training Grant  
**2019-** 2<sup>nd</sup> place poster presentation at the Cardiovascular Research Symposium - University of Louisville  
**2016-** 3<sup>rd</sup> best senior project presentation in the Biology Department – Nazareth College of Rochester  
**2015-** 1<sup>st</sup> place award for best presentation at CARS – Nazareth College of Rochester



## **RELATED EXPERIENCE**

**May 2015- August 2015** – Undergraduate Research Intern

My research revolved around concepts of environmental toxicology and chemistry in which I tackled a very relevant dilemma surrounding the US, the effects of hydraulic fracturing (hydro-fracking) and heavy metal toxicity associated with hydro-fracking. Under the guidance of Dr. Stephen Tajc, we were able to isolate and effectively utilize an organic bacterial byproduct capable of chelating toxic heavy metals from hydro-fracking wastewater solutions with efforts in bioremediation. During my time with Dr. Tajc, I traveled to numerous states in the U.S. such as California, Colorado, Georgia, and New York to publicly present my work in conference.

## **RESEARCH**

**2016-present** IPIBS Graduate Fellowship & NIEHS T-32 Trainee

Under mentorship of Dr. Daniel J. Conklin, my research focuses on the cardiotoxic and thrombotic effects that are associated with smoking conventional and electronic cigarettes, with special regards towards adverse platelet biological effects.

**2014-2016** Undergraduate Student Research

Under mentorship of Dr. Stephen Tajc, we investigated dipicolinic acid, a small molecule and organic compound, that is capable of chelating and extracting metal (II) cations from aqueous solution. This approach has implications for bioremediation of heavy metals in hydro-fracking wastewater.

## **PRESENTATIONS AND ABSTRACTS (order: Oldest to Most Recent)**

1. **Richardson, A.**, Porter, M., and Tajc, S. Extracting metal (II) cations from aqueous solution using dipicolinic acid. National American Chemical Society (ACS) Conference. Denver, CO, March 2015.
2. **Richardson, A.**, Porter, M., and Tajc, S. Extracting metal (II) cations from aqueous solution using dipicolinic acid. National American Chemical Society (ACS) Conference Regional at Genesee Community College. Genesee, NY, March 2015.
3. **Richardson, A.** and Tajc, S. Chelation of heavy metal (II) cations using 2,6-pyridine dicarboxylic acid. Creative Arts and Research Seminar (CARS). Rochester, NY, April 2015.
4. **Richardson, A.**, Sobraske, C., Porter, M., Luta, E., Page, K., and Tajc, S. Organic soil bacterial byproduct as a chelating agent in bioremediation of hydro-fracking wastewater. National American Chemical Society (ACS) Conference. San Diego, CA, March 2016.
5. **Richardson, A.**, Sobraske, C., and Tajc, S. Organic soil bacterial byproduct as a chelating agent in bioremediation of hydro-fracking wastewater. Creative Arts and Research Seminar (CARS). Rochester, NY, April 2016.

6. **Richardson, A.** and Tajc, S. Extracting metal (II) cations using dipicolinic acid with efforts to bioremediate hydro-fracking wastewater. Senior Comprehensive, Rochester, NY, May 2016.
7. **Richardson, A.** and Conklin, DJ. Harmful Tobacco Smoke Constituents and Cardiovascular Disease: New Tobacco Products, New Concerns? UofL Department of Pharmacology and Toxicology William J. Waddell Seminar Series. Louisville, KY, March 2017.
8. **Richardson, A.**, D'Souza, S., and Conklin, DJ. Electronic Cigarette Aerosol Aldehydes – Impact on Platelet Biology? Ohio Valley Society of Toxicology (OVSOT). Louisville, KY, July 2017.
9. **Richardson, A.**, D'Souza, S., and Conklin, DJ. Do Electronic Cigarette-Generated Aldehydes Affect Platelet Biology? Research! Louisville. Louisville, KY, September 2017.
10. **Richardson, A.**, D'Souza, S., and Conklin, DJ. Harmful and Potentially Harmful Constituents (HPHCs) and Cardiovascular Disease: Impact on Platelet Biology? UofL Diabetes & Obesity Center. Louisville, KY, October 2017.
11. **Richardson, A.**, D'Souza, S., and Conklin, DJ. Electronic Cigarettes and Aerosolized Aldehydes: Direct impact on Human Platelet Rich Plasma. Ohio Valley Society of Toxicology (OVSOT) Purdue University. West Lafayette, IN, December 2017.
12. **Richardson, A.** and Conklin, DJ. Investigating e-cig-generated aldehydes' influence on hemostasis. Post-doc presentation for the Diabetes and Obesity Center of the University of Louisville, Louisville, Kentucky, February 2018.
13. **Richardson, A.** and Conklin, DJ. Do Aldehydes in Electronic Cigarette Aerosols Stimulate Platelet-Mononuclear Cell Aggregation *in vivo*? Ohio Valley Society of Toxicology (OVSOT) Purdue University. West Lafayette, IN, June 2018.
14. **Richardson, A.** and Conklin, DJ. Do aldehydes in electronic cigarette smoke stimulate platelet-mononuclear cell aggregation *in vivo*? Abstract for poster presentation, Ohio Valley Society of Toxicology (OVSOT) at Purdue University, West Lafayette, Indiana, August 2018.
15. **Richardson, A.** and Conklin, DJ. Do Aldehydes in Mainstream Cigarette Smoke or Electronic Cigarette Aerosols Stimulate Platelet-Mononuclear Cell Aggregation *in vivo*? Research! Louisville. Louisville, KY, October 2018.
16. **Richardson, A.** and Conklin, DJ. Thrombosis: Stuck on tobacco or switch to e-cigarettes? Abstract for the University of Louisville Department of Pharmacology and Toxicology William J. Waddell Seminar Series, Louisville, Kentucky, October 2018.

17. **Richardson, A.** and Conklin, DJ. Thrombosis: Stuck on tobacco or switch to e-cigarettes? Post-doc presentation for the Diabetes and Obesity Center of the University of Louisville, Louisville, Kentucky, October 2018.
18. **Richardson, A.** and Conklin, DJ. Do aldehydes in electronic cigarette smoke stimulate platelet-mononuclear cell aggregation *in vivo*? Abstract for poster presentation, Ohio Valley Society of Toxicology (OVSOT) at University of Louisville, Louisville, Kentucky, November 2018.
19. **Richardson, A.** and Conklin, DJ. Tobacco-Derived Aerosols and Thrombosis. 1<sup>st</sup> Graduate Student Committee Meeting Presentation. University of Louisville, Louisville, Kentucky, December 2018.
20. **Richardson, A.** and Conklin, DJ. Smoking Out Unsaturated Aldehyde-Induced Platelet-Leukocyte Aggregation. Post-doc presentation for the Diabetes and Obesity Center of the University of Louisville, Louisville, Kentucky, April 2019.
21. **Richardson, A.** and Conklin, DJ. Do aldehydes in tobacco-derived aerosols stimulate platelet-mononuclear cell aggregation *in vivo*? Abstract for poster presentation. **Won 2<sup>nd</sup> place poster presenter.** Cardiovascular Research Symposium, University of Louisville, Louisville, Kentucky, April 2019.
22. **Richardson, A.**, Krivokhizhina, T., D'Souza, S., Srivastava, S., and Conklin, DJ. Phase-specific influences of electronic cigarette flavorings and acrolein on platelet aggregation. Abstract for poster presentation, 2019 Research! Louisville, Louisville, Kentucky, September 2019.
23. **Richardson, A.**, Krivokhizhina, T., D'Souza, S., Srivastava, S., and Conklin, DJ. Phase-specific influences of electronic cigarette flavorings and acrolein on platelet aggregation. Platform presentation for the Ohio Valley Society of Toxicology (OVSOT) meeting, Mason, Ohio, October 2019.
24. **Richardson, A.** Tobacco-derived aldehydes: platelet activation, thrombosis, and the role of TRPA1. M.S. Thesis/Ph.D. Proposal Presentation, University of Louisville, Louisville, Kentucky, November 2019.
25. **Richardson, A.** The effects of tobacco-derived harmful & potentially harmful constituents on platelet-leukocyte aggregation. Virtual presentation (due to COVID-19) for American Heart Association Tobacco Center for Regulatory Science (A-TRAC 2.0) Project 1 Teleconference, Louisville, Kentucky, April 2020.
26. **Richardson, A.**, Krivokhizhina, T., D'Souza, S., Srivastava, S., and Conklin, DJ. Effect of electronic cigarette flavorant additives on ADP-induced human platelet aggregation *ex vivo*. **Won 1<sup>st</sup> place graphical abstract presenter.** Graphical abstract for the Ohio Valley Society of Toxicology (OVSOT) summer meeting (virtual due to COVID-19), July 2020.

27. **Richardson, A.** Effects of tobacco flavorants on ADP-induced human platelet aggregation. Oral presentation (virtual due to COVID-19). University of Louisville Department of Pharmacology and Toxicology William J. Waddell Seminar Series, Louisville, Kentucky, October 2020.
28. **Richardson, A.** and Conklin, DJ. Do aldehydes in conventional tobacco smoke stimulate platelet-mononuclear cell aggregation *in vivo*? **Won 1<sup>st</sup> place Ph.D. poster presentation.** Abstract for poster presentation (virtual due to COVID-19), Ohio Valley Society of Toxicology (OVSOT), November 2020.
29. **Richardson, A.** and Conklin, DJ. Components of E-cigarette Liquids: Does GRAS Mean “Generally Regarded as Safe” or “Generally Regarded as Suspect”? (Adapted from Conklin, DJ). Virtual Presentation, February 2021.
30. **Richardson, A.** Pharmacology and Toxicology recruitment interview for prospective students. **Video interview** posted for YouTube. Louisville, Kentucky, March 2021.
31. **Richardson, A.** Prothrombotic Effects of Acute Exposures to Tobacco Product-Derived HPHCs on Platelet-Leukocyte Aggregation. Oral Presentation for the University of Louisville Diabetes & Obesity Center. Louisville, Kentucky, July 2021.
32. **Richardson, A.** Quick Pitch: Smoking and Platelet Activation. **Won 1<sup>st</sup> place oral elevator pitch.** Trainee Retreat, Louisville, KY, July 2021.
33. **Richardson, A.** What is the mechanism(s) behind cigarette smoke-induced platelet activation? 3-minute oral and graphical presentation (virtual due to COVID-19), Ohio Valley Society of Toxicology (OVSOT) summer meeting, July 2021.
34. **Richardson, A.** and Conklin, DJ. Cigarette Smoke-Induced Platelet Activation: Acute Effects of Aldehydes *in vivo*. Abstract for poster presentation, 2021 Research! Louisville, Louisville, Kentucky, October 2021.
35. **Richardson, A.** Cigarette Smoke-Induced Platelet Activation: A TRPA1-Dependent Effect? University of Louisville Department of Pharmacology and Toxicology William J. Waddell Seminar Series, Louisville, Kentucky, October 2021.
36. **Richardson, A.** and Conklin, DJ. Cigarette Smoke-Induced Platelet Activation: Acute Effects of Aldehydes *in vivo*. Abstract for poster presentation (virtual due to COVID-19), Ohio Valley Society of Toxicology (OVSOT), November 2021.
37. **Richardson, A.\***, Krivokhizhina, T.\*, Lorkiewicz, P., D’Souza, S., Bhatnagar, A., Srivastava, S., and Conklin, DJ. Effects of Electronic Cigarette Flavorant Additives on Human Platelet Aggregation *ex vivo*. Abstract for poster presentation, 2022 Annual Society of Toxicology (SOT) meeting, San Diego, CA, March 2022.

38. **Richardson, A.** and Conklin, DJ. Tobacco-derived aldehydes, platelets, and thrombogenicity: role of transient receptor potential ankyrin-1. Mock #1 Ph.D. Dissertation Defense for Diabetes and Obesity Center, Louisville. Kentucky, May 2022.

39. **Richardson, A.** and Conklin, DJ. Tobacco-derived aldehydes, platelets, and thrombogenicity: role of transient receptor potential ankyrin-1. Mock #2 Ph.D. Dissertation Defense and job interview at Center for Toxicology and Environmental Health (CTEH), North Little Rock, AR, June 2022.

39. **Richardson, A.** and Conklin, DJ. Tobacco-derived aldehydes, platelets, and thrombogenicity: role of transient receptor potential ankyrin-1. Ph.D. Dissertation Defense, Louisville. Kentucky, June 2022. **Successfully Defended.**

### **MANUSCRIPTS/PUBLICATIONS**

1. Lynch, J., Jin, L., **Richardson, A.**, Jagatheesan, G., Lorkiewicz, P., Xie, Z., Theis, W., Shirk, G., Malovichko, M., Bhatnagar, A., Srivastava, S., and Conklin, DJ. Acute and chronic vascular effects of inhaled crotonaldehyde in mice: role of TRPA1. *Toxicology and Applied Pharmacology*. 2020.

2. Lynch, J., Jin, L., **Richardson, A.**, and Conklin, DJ. Tobacco smoke and endothelial dysfunction: role of aldehydes? *Current Hypertensions Report Review*. 2020.

3. Jin, L., Lynch, J., **Richardson, A.**, Lorkiewicz, P., Theis, W., Shirk, G., Hand, A., Srivastava, S., Bhatnagar, A., and Conklin, DJ. Electronic cigarette solvents, pulmonary irritants and endothelial dysfunction: role of acetaldehyde and formaldehyde. *American Journal of Physiology: Heart and Circulatory Physiology*. 2021.

4. **Richardson, A.\***, Krivokhizhina, T.\*, Lorkiewicz, P., D'Souza, S., Bhatnagar, A., Srivastava, S., and Conklin, DJ. Effects of tobacco flavorants on ADP-induced human platelet aggregation ex vivo. *Toxicology Reports*. 2022

### **PROFESSIONAL SOCIETIES**

**2021-present** Society of Toxicology (SOT) graduate student member

**2021-present** Board of Directors of the West Jefferson County Community Task Force (WJCTF; Profile Link: <https://wjctf.org/board-of-directors/>)

**2017-present** Ohio Valley Society of Toxicology (OVSOT)

**2013-2016** American Chemical Society (ACS)

### **EXTRACURRICULAR ACTIVITIES**

**2016-2019** Coach of the Louisville FURY Boys Volleyball Club

**2016-2018** Volunteer Assistant and Practice Player of University of Louisville NCAA Division I Women's Volleyball Team

**2014-2016** Nazareth College Division III Men's Volleyball Student-Athlete  
Our 2015 team were NCAA Division III Men's Volleyball National Semifinalist, finishing 3<sup>rd</sup> national ranking in the final AVCA coaches' poll.