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Confessions of the Heart: A Study Focusing on the Role Electronic Cigarettes Play in Modulating Heart Rate Variability in C57BL/6 Mice

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by

Jocelyn Andrea Castellanos

2020

ABSTRACT OF THE THESIS

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Jocelyn Andrea Castellanos

Master of Science in Environmental Health Sciences

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Professor Jesus A. Araujo, Chair

Over the last decade, e-cigarette (EC) use has increased rapidly, garnering widespread attention, particularly in teens and young adults. In addition, with its perceived assumption of safety bolstered by controversial findings indicating ECs toxicants to be found at lower levels than in tobacco cigarettes (TCs), ECs have spurred much debate in public health about its role in contributing to adverse health effects. However, while multiple studies support a causal link between tobacco cigarette (TC) use and a decline in health, there is a paucity of data with EC use

in particular with cardiovascular (CV) health. Furthermore, previous studies have primarily focused on CV vital signs (i.e.: heart rate and blood pressure) using varying nicotine concentrations, flavors and EC designs thereby contributing to EC polemics. Since no empirical data are available for the increased risk of CV disease from EC exposure, we must examine CV health from a different perspective. Indeed, one human study assessing heart rate variability (HRV) found increased sympathetic activation (cardiac autonomic dysregulation) in "chronic" EC users, a biomarker known to indicate reduced cardiovascular health from TC exposure. However, EC-mediated alteration in heart rate variability has not been studied in an acute setting. In addition, no animal models exist with respect to HRV analysis and EC use that take into consideration human vaping topography like puff duration, inter-puff interval, and the episodic nature of EC use. Therefore, we aimed to investigate the role acute EC exposures play in changing heart rate variability using an in-vivo exposure system in which real-life conditions could be reflected.

Telemetry devices were implanted in the abdomen of six eight-week-old C57BL/6 mice to monitor electrocardiographic activity continuously. Mice underwent a 1-hour acclimation phase in exposure chambers followed by a 1-hour exposure to air (control). E-cigarette exposures consisted of two 15-minute sessions (4-sec puff/26-sec air) to a BluPLUS+ device containing 2.4% nicotine with Classic Tobacco flavoring. To validate previous studies using the same exposure system, a 15-minute period of aerosolized phosphate-buffered saline (PBS) was added as a secondary control. For each exposure episode, a 45-minute post-exposure event followed. Ponemah v6.20 software was used for heart rate variability analysis of the time and frequency domain. Additionally, real-time particle number and mass concentrations as well as size distributions were monitored with a scanning mobility particle sizer and an aerodynamic particle

sizer for particles within the 7.37 – 289 nm and 0.5 – 19.8 µm size ranges, respectively. In addition to real-time measurement, a personal cascade impactor was used to measure particulate matter with an aerodynamic diameter of 2.5 or below ($PM_{2.5}$) mass concentration during a 15-minute exposure period following the gravimetric method.

TSI Data Merge Software Module (TSI Inc., Shoreview, MN), which converts electronic mobility diameter measured by the SMPS to aerodynamic diameter was used to merge SMPS data with APS resulting in composite size distributions. Aerosol data was then plotted using normalized concentrations (dN/dlogDp) to correct for differences between instruments and provide a best fit for single source aerosols. Frequency domain parameters were log-transformed and a linear mixed-effects model with a robust estimator was used to determine the relationship between EC events vs. air and PBS and frequency parameters were log-transformed. R (Version R3.6.3) was utilized to test for statistical significance at $\alpha = 0.05$.

Size distributions and temporal profiles of particle number and mass concentration analysis revealed the ability for the EC aerosol exposure system to effectively deliver EC aerosol in an acute manner. Additionally, the detection of a bimodal distribution of ultrafine and fine particles support previous research and may indicate these particles were generated from pyrolysis, metals and volatile organic compounds. Furthermore, high levels of $PM_{2.5}$ levels (8072 $\mu g/m^3$) were detected during a 15-minute exposure period. With respect to HRV, the standard deviation of normal sinus beats (SDNN) and the root mean square of successive differences between normal heartbeats (RMSSD) were analyzed to measure non-physiological changes of autonomic variability. EC exposures significantly decreased SDNN and RMSSD by 5.00 ($CI_{95\%}$ [-8.28, -1.23]) and 9.34 ($CI_{95\%}$ [-15.01, -3.67]) units as compared to air exposures, suggesting reductions in total and short-term autonomic variability. In contrast, the proportion of

normal consecutive heartbeats differing by six, (pNN_6) was elevated (14.72, $CI_{95\%}$ [11.96, 17.47]). However, further studies are needed to corroborate the reliability of this measure.

Frequency domain parameters were also analyzed to determine physiological changes in HRV. Such parameters included in this study were low frequency (LF), an indicator suggestive of sympathetic activity, high frequency (HF), a marker for sympathetic tone, and the ratio of the LF to HF (LF/HF), a parameter reflecting sympathovagal balance. Significant increases in log transformed low frequency (LF) by 1.33 units ($CI_{95\%}$ [1.14, 1.52]),) were also observed. Results for the log (HF) parameter (0.51, $CI_{95\%}$ [0.28, 0.73]) also indicated the presence of the parasympathetic branch during the EC exposures, but the magnitude was less than that of LF. Even so, log (LF/HF) by 0.11 units during EC exposure in comparison to combined air suggest parasympathetic predominance. However, this measure may not be a true indicator of the balance between the sympathetic and parasympathetic branches. Furthermore, when EC exposures were compared to PBS, differences were more striking in almost all HRV parameters. Moreover, when looking at individual EC exposures, larger differences were observed during the second EC exposure episode than the first which may be important for chronic exposure assessment. In short, this study demonstrates that acute ECs have the potential to induce changes to HRV which may have implications in autonomic dysregulation.

The thesis of Jocelyn Castellanos is approved.

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TABLE OF CONTENTS

LIST OF FIGURES X
LIST OF TABLES XI
1. INTRODUCTION 1
1.1 The Rise of ECs 1
1.2 Hypothesis and Objectives 3
2. BACKGROUND 4
2.1 What are ECs Made of? 4
2.2 Potential Alternative to Smoking Aid 5
2.3 EC Chemical Composition and Their Potential Health Impact 7
2.3.1 E-liquid Composition 8
2.3.2 EC Emissions and Unintended Contaminants 11
2.4 ECs and Cardiovascular Health 15
2.4.1 Acute EC Exposure 16
2.4.2 Chronic EC Exposure 17
2.5 Assessing CV Health 17
2.5.1 HRV as an Indicator for CV Health 18
2.6 Testing Models 20
3. MATERIALS AND METHODS 21
3.1 C57BL/6 Mice 21
3.2 Chamber Set up 21
3.3 Data Acquisition and HRV Analysis 23
3.3.1 Time Domain Measures 24
3.3.2 Frequency Domain Measures 24
3.4 Aerosol Characterization 25
3.5 Statistical Analysis 26
4. RESULTS 27
4.1 Aerosol Characterization 27
4.2 Physiological Characteristics 29
4.3 Time Domain Analysis 31
4.4 Frequency Domain Analysis 34
5. DISCUSSION 37

5.1 Experimental Design and Vaping Topography Considerations 37
5.2 PM and Potential Health Impacts 39
5.3 Changes in Heart Rate Variability 41
5.4 HRV and Experimental Design Considerations 43
5.5 Limitations 45
6. CONCLUSION 47
REFERENCES 49

LIST OF FIGURES

Figure 1. Components of a First-Generation E-cigarette.....4

Figure 2. E-cigarette Aerosol Generation System 22

Figure 3. Schematic of the Experimental Design 23

Figure 4. Time Series Plots of Total EC Particle Number and Mass Concentrations 28

Figure 5. Normalized Particle Size and Mass Distributions 29

Figure 6. HRV Reductions Measured During EC Exposure in Time Domain Parameters 32

Figure 7. Marginal Effects of Log- transformed Frequency Domain Parameters. 35

LIST OF TABLES

Table 1. Physiological Parameters of C47BL/6 mice 30

Table 2. Custom Contrasts for Time Domain Parameters 33

Table 3. Custom Contrasts for Frequency Domain Parameters 36

1. INTRODUCTION

1.1 The Rise of ECs

Since its introduction in 2007, electronic cigarettes (ECs) have exploded onto the tobacco product market. In 2011, quarterly e-cigarette retail sales (excluding online and vape shops sales) increased from approximately $19 million to $409 million by the end of 2017. Aggregated annual EC retail sales increased 16% in 2015-2016 and 47% in 2016-2017, with more than 58 million ECs and refills sold in the United States at grocery and convenience stores[1,2]. Furthermore, recent data showed that ECs accounted for 3.6 billion dollars in the U.S. in 2018[3] and the prevalence of vape shops surged catering to the fast-growing EC market[4,5]. While its initial formation was to provide smokers a "safe" alternative for tobacco smoking products, this expanding EC market does not just target smokers specifically.

An estimated 10.8 million (4.5%) adults and 3.05 million (20.8%) high school students in the states are current EC users, a prevalence that has been described by the U.S. Food and Drug Administration (FDA) as "an epidemic"[3]. More specifically, a recent study conducted by the Center for Disease Control and Prevention (CDC) determined that more than 5 million U.S. students used ECs within the past 30 days, including 10.5% of middle school and 27.5% of high school students[6], demonstrating youth rather than adults are more likely to use ECs. Yet, the question is why? Is this some kind of scheme that the "Big Tobacco" industry has created to addict the next generation to nicotine at a time when tobacco (TC) smoking was finally reducing? Well, according to a survey conducted by Villanti et. al., compelling advertisements that provide a variety of juice flavors and easy-to-use accessories have been cited as the primary reason for vaping in 81% of current youth[7]. So, while the verdict is still out on this particular

subject, from a public health perspective, the increasing rates among young individuals is highly concerning.

ECs have garnered widespread attention in the last decade or so particularly due to its unprecedented popularity, specifically with minors. While they come in many shapes and sizes, all ECs have the same elemental components: a lithium-ion battery, a heating element and pod or tank that holds liquid. Its initial introduction into the market was to simulate the experience of smoking tobacco, and early designs mimicked regular TCs[8]. Thus, while ECs produce an aerosol inhaled by the user like TCs, they differ in the method by which it is created. TC users inhale an aerosol produced by the incomplete combustion of tobacco and generate thousands of chemical substances via distillation, pyrolysis and pyrosynthesis[9]. ECs, on the other hand, produce aerosols by heating a liquid containing propylene glycol, glycerin, water, flavorings, and nicotine in various concentrations[10]. Most of these components are considered harmless and used as additives in other foods, spurring the assumption that ECs are safe. Yet, according to the U.S. Department of Health and Human Services, EC aerosol can also contain a number of potentially harmful substances including nicotine, ultrafine particles, flavorings such as diacetyl, volatile organic compounds, cancer-causing chemicals, and heavy metals such as nickel, tin, and lead[11]. However, scientists are only beginning to scratch the surface of its potential health impacts.

In particular, while TCs and ECs are vastly different entities, their rise in the market and route of exposure are similar. TC smoke has been indicted as a risk factor in many cardiovascular (CV) diseases like coronary artery disease (CAD), stroke, and aortic aneurysms[12,13]. Recent studies looking at the potential for ECs to induce CV risk have studied their role through the lens of cardiovascular vital signs (i.e., blood pressure and heart rate) with conflicting results. Therefore, the probability that ECs may induce CV diseases must also be

assessed from data gathered on constituent toxicity, exposure levels, mechanisms and experimental model studies from known data on TC- induced CV harm[13]. One such study assessing sympathetic nerve activation, a mechanism predictive of CVD in TC smokers by, in EC vapers determined that chronic EC use was associated with a shift in sympathovagal balance towards sympathetic predominance[3,14]. As increased sympathetic nerve activity measured by heart rate variability (HRV) is associated with increased CV mortality[3,14,15], further research is warranted to determine the role ECs play in CV health.

In summary, the potential for minors to form new habits of vaping nicotine-containing ECs and become regular users or transition to tobacco smoking is an issue that cannot be ignored. Unfortunately, ECs are relatively new and while much research has been conducted since its debut more than a decade ago, we are still learning about their potential health impacts. Moreover, with its comparable design and mode of transmission (inhalation) to TCs, it is imperative to understand whether ECs may use similar mechanisms by which conventional cigarettes cause illness such as cardiovascular disease. The following sections will outline what is known about ECs from its make up to the potential health impacts users may face.

1.2 Hypothesis and Objectives

With a veritable lack of data concerning ECs and HRV, this study aimed to develop a sensitive mouse model to evaluate acute effects resulting from electronic cigarette exposures and test for potential CV effects associated with cardiac autonomic regulation. Based on previous human data in which EC chronic users exhibited measures suggestive of decreased heart rate variability, this study hypothesized acute EC exposure would also lead to autonomic dysregulation via reductions in HRV in freely moving C57BL/6 mice.

2. BACKGROUND

2.1 What are ECs Made of?

As previously mentioned, ECs come in different shapes, sizes, classifications, and styles. All current ECs - also commonly known as electronic nicotine delivery systems (ENDS) - contain a lithium-ion battery, atomizer or cartomizer, and a cartridge to hold the liquid; all wrapped in one enticing cover composed of various metals and plastics[10] (**Figure 1**). When a sensor in the device detects airflow, the heating component is activated and vaporizes the liquid (also known as e-liquid) solution producing a smoke-like aerosol, which is then inhaled[10,16-19]. Advanced devices function in the same method, however, power is applied when the user presses a button. Depending on the size of the product, the amount of aerosol delivered is driven by the battery's capacity[20]. Small ECs can deliver the contents of a single, prefilled cartridge- approximately 1mL of liquid on a single charge. Larger devices can deliver 5-40mL of content prior to charging.

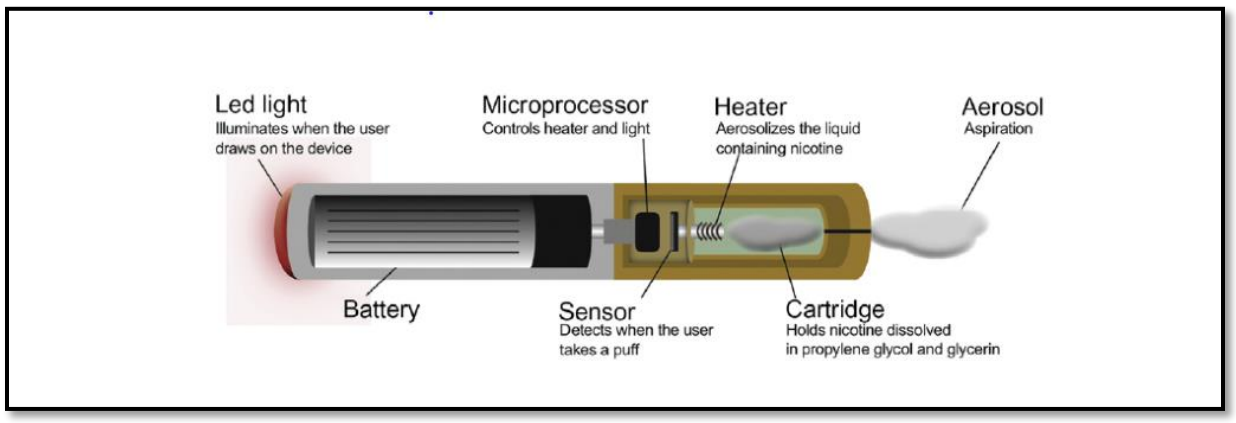

Figure 1. Components of a First- Generation Electronic Cigarette.

As is obvious, ECs include a very diverse line of products, with different designs, functionalities and even performance features. While ECs go by many names, products available on the market can be classified by generation. First-generation devices mainly called "cigalikes"

due to their resemblance to TCs, are rather simplistic with a battery and cartomizer. The battery can either be disposable or rechargeable. Second-generation devices consist of a rechargeable lithium battery of larger size, resembling a pen[20]. Third-generation ECs called "mods" or "advanced personal vaporizers (APV) contain large lithium batteries with integrated circuits allowing the user to adjust energy delivery to the atomizer. Lastly, fourth-generation devices are called "pod mods" and are similar with respect to battery and adjustability to third-generation ECs. Common pod mod brands typically use nicotine salts which have a lower pH than free-base nicotine, allowing for higher nicotine levels to be inhaled[21].

While cigalikes resemble TCs in shape, form, weight, and function, they have low aerosol volume production and low nicotine delivery potential[22,23]. Steadfast users showed a preference for newer generation ECs as they provided better sensory satisfaction and more nicotine delivery comparable to TCs[19,24-27]. Still, a wide variety of liquids are available with thousands of flavors and nicotine content including non-nicotine e-liquids.

2.2 Potential Alternative to Smoking Aid

ECs have incited dramatically divergent reactions among public and healthcare officials- embraced as possible replacements for lethal TCs or disparaged as a means to addict the next generation to nicotine. However, it is important to holistically understand both sides of this story to later inform policies and regulations. While abstaining from smoking is known to lower the risk of developing diseases and produce significant health gains in patients[28,29], most smokers that want to quit find it difficult. Most attempts at quitting end in failure largely due to the powerful addictive qualities of nicotine as well as non-nicotine sensory and behavioral cues[30,31]. Outside of programs that provide a combination of pharmacotherapy and behavioral intervention,

the cessation rates are extremely low (estimated annual population rate was 4-5% in 2017)[20]. Thus, the need for alternative tools are required.

This introduces the idea of ECs as potential tobacco harm reduction products. While earlier population studies demonstrated that regular use of ECs occurred mostly among smokers and former smokers, a recent study showed that many adults using ECs in an attempt to quit smoking did not stop smoking cigarettes and instead continued to use both products (i.e., dual use)[3]. In fact, between 2015 and 2018, the prevalence of current EC use did not change significantly among current smokers (29.8% in 2015; 27.7% in 2018) providing evidence of continued dual use. Furthermore, among never ($p = 0.012$) and former ($p<0.001$) smokers the prevalence of current EC use increased significantly within a three-year span[32], disputing the thought of ECs as tobacco harm-reduction products. However, Owusu and colleagues did find that among current EC users, cigarette smoking significantly declined from 56.9% to 40.8% ($p<0.001$)[33]. As such, it is reasonable to suggest that ECs have a potential to benefit current smokers as a cessation aid, but also raises the prospect for never and former smokers to initiate smoking other tobacco-related products or relapse.

Another key component of the effectiveness of ECs as a smoking cessation tool is the role of nicotine. Major health organizations such as the U.S. FDA have accepted long-term nicotine replacement therapies (NRTs) as viable substitutes for TC smokers[20,34-36]. From what we understand, nicotine has known health effects (e.g. highly addictive nature, toxicant to developing fetuses, can harm brain development in adolescent individuals continuing into the mid-20's)[11], however several studies evaluating the effects of noncombustible nicotine products have shown it is highly unlikely for these products to significantly contribute to smoking-related cancer and cardiovascular disease[20].

With respect to ECs, initial randomized control trials (RCTs) using first-generation ECs (less effective nicotine delivery devices compared to subsequent generations) showed that at six months, the abstinence rate for EC groups with or without nicotine were similar to the nicotine patch[3]. Furthermore, Rahman et. al, found that ECs with nicotine were more effective as cessation tools than non-nicotine ECs; however, there was insufficient data to compare ECs to certified U.S. FDA approved cessation strategies[37]. Only recently data has become available in demonstrating ECs as superior to traditional NRTs. A multicenter, randomized trial with 889 TC smokers enrolled found that 80% of individuals who successfully quit TC smoking were still using their assigned EC product after a one-year abstinence rate and only 9% of NRT users were still using assigned nicotine replacement products[3]. This emerging evidence points to the concept that ECs are effective TC cessation devices over NRTs, however TC smokers have largely switched to ECs, resulting in long-term EC use rather than actual cessation of all nicotine products. In summary, EC's role as a harm-reduction product is not so straightforward. Thus, we must consider the possible health risks users may face, particularly in never-before smokers like minors and former TC smokers as they make decisions to begin utilizing ECs.

2.3 EC Chemical Composition and Their Potential Health Impact

It is important to understand not only the functionality and various EC types used, but also the composition of the liquid and aerosol to better gauge possible risks from EC exposure. The main constituents in e-liquid are propylene glycol (PG), glycerin (GLY), nicotine, and various flavorings. However, unintended contaminants such as tobacco-specific nitrosamines (TSNAs) may form from the primary chemicals. Thermal decomposition products formed during the heating process such as aldehydes, and leachable materials that transfer from the device or

tank like plasticizers and metals have, additionally, been found in EC liquids or aerosol[20,27]. Others have reported the detection of compounds unlikely to form from pyrolysis (e.g. polyaromatic hydrocarbons- PAHs) in tobacco-flavored liquids[38,39]. This section will outline the various EC chemical constituents and possible health impacts from exposure.

2.3.1 E-liquid Composition

2.3.1.1 Propylene Glycol:

In ECs, PG is widely used due to its solvent properties and production of a visible aerosol when heated (0 – 82.875 mg/15 puffs)[40]. PG is mainly responsible for throat irritation or rather "throat hit", a desired effect for both smokers and EC users[41,42]. PG has also been cited as a carcinogen, ocular irritant, gastric distress product, and found to increase asthma risk in children[12,13]. As indicated by Farsalinos, PG is not expected to be of concern when absorbed systemically because of the body's ability to metabolize it in addition to low daily consumption of the product[20]. However, with newer generation of ECs giving users control on how much liquid is aerosolized and exhaled with variable voltage, it stands to reason that propylene glycol does pose a risk for human health if taken in higher doses.

2.3.1.2 Glycerin:

GLY is mainly used in ECs for similar reasons as stated with PG. However, it is thought to cause a milder form of "throat hit" and is more commonly used in higher volumes in low or non-nicotine liquids with direct passage into the lungs during inhalation[20]. As the systematic absorption of GLY appears to be innocuous and little evidence raises concerns or demonstrates adverse health effects, GLY seems safe. Chaumont et al., found that acute vaping of PG/GLY aerosol at high voltages with or without nicotine induced airway epithelium injury and decreased transcutaneous oxygen tension in young tobacco smokers[43]. Of course, with a small number of

tobacco-only smokers, the results may seem insubstantial. Regardless, it is important to note that while the outcome is not from GLY exposure alone, future respiratory issues may be primarily driven from PG/GLY subjection rather than nicotine and spurs the idea that together, these substances may not be as safe as previously thought.

2.3.1.3 Nicotine:

Almost all ENDS deliver nicotine. This compound is particularly known for its addictive properties causing either ganglionic stimulation in low doses or ganglionic blockage in high doses in the central nervous system (CNS)[44.] In addition, nicotine's ability to stimulate the CNS can induce the release of neurotransmitters (e.g. acetylcholine, beta-endorphin, dopamine, norepinephrine, and serotonin) and adrenocorticotropic hormone (ACTH) resulting in peripheral vasoconstriction, tachycardia, elevated blood pressure and vomiting or nausea[45].

Indeed, various studies have evaluated biomarkers of nicotine uptake and found that e-cigarette users were able to attain nicotine and cotinine concentrations comparable to cigarette smokers after evaluation of plasma nicotine, plasma or serum cotinine, and salivary cotinine[45,46]. In addition, salivary cotinine concentrations in former smokers using ECs daily, reached and maintained levels of cotinine observed in TC smokers over an 8-month period by varying their consumption of e-liquid[47]. In another study conducted on heavy tobacco smokers who abstained for 6 hours prior to EC use, plasma nicotine and cotinine levels showed that ECs delivered nicotine effectively, although the pharmacokinetic profiles differed in comparison to TCs. This suggests that nicotine uptake in the plasma of EC users are high enough to produce and maintain nicotine dependence, especially in young individuals. Moreover, this may explain the potential for adolescents to transition to tobacco smoking in adulthood as the U.S Surgeon General Report indicated in 2016[11].

As previously mentioned, most ECs contain nicotine and much of the nicotine employed is derived from tobacco plants. While, tobacco-based nicotine is highly purified via extraction and distillation, it still contains certain impurities. In particular, Goniewicz et al. detected N-nitrosonornicotine (NNN) and 4-(methylnitrosamino)1-(3-pyridyl)-1-butanone (NNK) in EC aerosol. Concentrations ranged from 0.8 - 4.3 ng/150 puffs (NNN) and 1.1 - 28.3 ng/ 150 puffs (NNK)[48]. In conjunction, Farsalinos et al. discovered 7.7 ng/g of NNN and 2.3 ng/g of N-nitrosanabasine (NAB) in EC liquid, however, NNK was not found in samples tested[39]. The issue with the detection of these chemicals, is that both NNN and NNK are known carcinogens to humans and recognized as such by the International Agency for Research in Cancer (IARC)[49]. It is thought that TSNAs present in e-liquids may be imparted due to the use of other tobacco-derived ingredients (flavors extracted from tobacco) or materials in productions[20], but more research is required to determine the impact of such sources.

2.3.1.4 Flavorings:

Flavorings play an essential role in ENDS as they are almost flavorless without these additives. Various surveys have stated the importance of flavorings in the use of ECs for consumers; approximately 99% of users utilize some form of flavored liquids and on average can use up to three different kinds a day[50]. In fact, fruit flavors were commonly cited as popular throughout the course of usage, with tobacco flavors popular during initial use in adults[50]. The majority of young "vapers" reported initiating EC use with flavored varieties and implicated them as the primary reason for operating ECs[50].

While there are many flavor compounds available in the market considerable efforts has been given to diacetyl (DA) and acetyl propionyl (AP). DA has been implicated in the development of bronchiolitis obliterans, otherwise known as "popcorn lung disease",

appropriately named given the ailment was first noted in popcorn factory workers[51]. While the condition is rare, popcorn lung disease is life-threatening and a nonreversible form of obstructive lung disease. With respect to ECs, a 2014 investigation[51] examining 159 "ready to use" and concentrated flavored e-liquids from 36 manufactures across the U.S. and Europe, detected either compound in 74.2% of samples. A separate study[52] corroborated somewhat similar findings when evaluating 51 ECs. On average, concentration ranges (0.3 - 329 µg/EC; 0.2 – 64 µg/EC) were much lower in this study; samples were collected until EC emissions was no longer visible which do not paint a realistic usage pattern.

Other flavors detected in ECs have also been studied with varying success. Cinnamaldehyde, a popular substance found in cinnamon-flavored liquids, has been shown to be cytotoxic in vitro, however, no empirical data are available to show the possible health risks EC users may face[20,50]. Lastly, tobacco-extracted flavors are produced from whole tobacco leaves and the process may transfer unwanted chemicals. Although the evidence is limited, some extracts contain elevated levels of phenolic compounds and nitrate, potentially including PAHs and other combustible products. However, this possibility may only arise if the source tobacco was cured with wood smoke[53].

2.3.2 EC Emissions and Unintended Contaminants

2.3.2.1 Particulate Matter:

Another health concern related to ECs is the generation of fine and ultrafine particles. Inhalation of particulate matter originating from the combustion of fossil fuels have been known to contribute to adverse health impacts and it is understood that no concentration level is safe in which no adverse effects are anticipated[54]. In particular, due to their small size they can penetrate airways and deposit deeper into the lungs or reach the circulation more easily leading to adverse

lung and cardiovascular effects[56]. Aerosols with an aerodynamic diameter of 2.5 micron or less are considered fine particles ($PM_{2.5}$) whereas aerosols with an aerodynamic diameter of 0.1 micron or less are considered ultrafine ($PM_{0.1}$). While both are of major concern, this segment will refer to EC in relation to $PM_{2.5}$ as most studies have focused on fine particles.

Previous studies evaluating their presence in EC aerosol detected significant levels of $PM_{2.5}$ exhaled by the user[12,55]. This was comparable to TC smokers, although the number of particle and mass distribution varied depending on puff topography, nicotine concentration and e-liquid[56-58]. Additionally, second- and third-hand smoke have also been detected from EC aerosol. Several studies mimicking real-life settings demonstrated significant increases in $PM_{2.5}$ concentrations in rooms or experimental chambers from exhaled EC aerosols, highlighting ECs as a source of secondhand exposures[12]. Other studies provide evidence that thirdhand exposure adversely affects indoor air quality with the potential to release potentially noxious substances[56,57]. In tobacco smoke studies, secondhand and thirdhand exposures are known to exert toxicity on various biological systems[57], therefore these exposures originating from ECs may pose a problem not only to the user but to non-users as well. However, little to no studies have examined the biological effects of particulate matter generated by ECs, therefore further research is needed within this field.

2.3.2.2 Aldehydes:

During the heating of PG and GLY, thermal dehydration compounds are produced, namely aldehydes. Specifically, GLY has historically been known to produce aldehydes such as acrolein, formaldehyde, and acetaldehyde[20,59,60]. The generation of these chemicals is one concern given the known health effects. Formaldehyde and acetaldehyde are classified by the IARC as human carcinogens[61] with implications in mediating bronchitis, pneumonia, and asthma

risk in children from formaldehyde exposure. In addition, this substance is a known ocular, nasal and throat irritant while acetaldehyde has shown to exacerbate alcohol-induced liver damage[12]. Acrolein has also been indicted as a nasal irritant and known to damage the lining of lungs[62].

Due to the nature of ECs, generation of various aldehydes is highly dependent on temperature provided and in turn, battery voltage by which it supplies power to the atomizer. An increase in voltage from 3.3 volts (V) to 4.8V can double the amount of e-liquid vaporized and increase the total aldehyde fornation[20,12,13]. Thus, at low battery wattage aldehyde emissions are relatively low compared to TCs, but at high voltages, emissions are closer to and could exceed those generated by TCs[13]. Reuse of devices can also increase aldehyde formation via the buildup of polymers that degrade when heated[13]. However, some researchers suggest that at these high temperatures, taste of the aerosol would be compromised and thus reduces the possibility that users would increase battery power to elevated settings[63]. Therefore, we must consider development of environments that simulate EC use behavior when extrapolating laboratory studies of emissions to human disease risk.

2.3.2.3 Free Radical Formation:

While the formation of highly reactive, short-lived free radicals and stable long-lived free radicals have been discerned, the chemical nature of the radicals remains unclear[12]. However, investigators have discovered that reactive free radicals generated by ECs are 100-fold to 1,000-fold lower than TCs, but daily exposures to free radicals from regular EC exposure is estimated to be higher than those generated by air pollution, a known parameter for increased cardiovascular risk[12]. Furthermore, mechanisms by which free radicals may form from EC exposure are not fully understood.

Certain free radicals have the potential to cause oxidative stress (an imbalance in which oxygen radicals cannot be gradually destroyed leading to an overload of these species) in the body, thereby inducing the development of certain infirmities like cancer, autoimmune disorders, aging, cataracts, rheumatoid arthritis, neurodegenerative and cardiovascular diseases[64]. Only recently have findings begun to implicate the role EC plays in oxidative stress. Kuntin and colleagues determined that e-cigarette exposure increases vascular, cerebral, and pulmonary oxidative stress in mice when exposed to EC vapor via a phagocytic NADPH-oxidase-dependent mechanim[65]. Interestingly, they found acrolein to be the key mediator in this pathway giving credence to the significant oxidative potential aldehydes have[3].

2.3.2.4 Metals:

Before or during the formation of EC aerosol, contaminants introduced from the device may leach into the e-liquid. As the heating coil and tank are often constructed of metal, it is hardly surprising that they are the main leachable material. As such, concerns for metal exposure are derived from serious health effects associated with them like neurotoxicity[66] and cardiovascular disease[67] from lead, and respiratory illness[68] and lung cancer from chromium and nickel[69,70]. Goniewicz et al.[48] reported cadmium, chromium, nickel, and arsenic in sampled aerosol, while Williams and colleagues[37] reported tin, silver, iron, nickel aluminum, and silicate particles larger than 1 micron. They also found tin, chromium and nickel nanoparticles of less than 0.10 micron in EC aerosol. While these investigators used first-generation ECs, recent studies support these initial findings[71,72] and determined that chromium, nickel, and lead exceeded the current health-based limits by 50%[73]. Further investigations are required, however, specifically, with the testing of other generations of ECs, in addition to understanding the potential mechanisms by which toxic metals may promote disease.

In light of this, analyses of e-cigarette liquids and vapors have shown generally lower levels of many of the toxicants found in cigarette smoke. In addition, because ENDS do not result in the direct combustion of organic carbonate materials known to have adverse health effects like conventional cigarettes, this further spurs the assumption of safety. However, that may not be necessarily true. As previously outlined, an increasing body of published studies to date demonstrate the prospective health risks associated with EC constituents themselves. Moreover, growing evidence points to the dangers unintended contaminants may have on EC users, however, we cannot yet draw causal inference. Further research is required in the evaluation of not only these toxicants but also the mechanism by which they may produce health effects.

2.4 ECs and Cardiovascular Health

EC ingredients have the potential for inducing health risks as previously mentioned; however, limited studies exist. Given the important causal link between TC smoking and CV disease (CVD)[73-75] and their similar modes of exposure, it is paramount to study these products within the realm of CV health.

CVD is the leading cause of death globally representing 31% of all mortality, 85% of which are due to heart attack and stroke[76]. Most cardiovascular diseases can be prevented by addressing behavioral risks such as TC use, unhealthy diet, physical inactivity and use of alcohol. Among smokers, CVD is a major cause of preventable death, accounting for as much as 30% of heart disease-related mortality in the U.S. each year[20,12,77]. Mortality from smoking occurs via damage to the heart and blood vessels, increasing the risk for heart conditions such as

atherosclerosis, stroke, heart attack, aortic aneurysm, and peripheral arterial disease[77,78]. However, little is known on the role ECs may have with respect to cardiovascular disease.

In regard to the debate of whether EC use may be a benefit or risk to human health, the cardiovascular system is an important consideration. As previously discussed, EC emissions may have negative effects in CV health via exposure to nicotine, aldehydes, particulates and flavorings[13]. Furthermore, the justification of using ECs because of their "lower" levels of harmful constituents conferring their "safe" status, must be reviewed. It has been shown that TCs have a nonlinear dose response relationship with cardiovascular risk, therefore, even exposure to low levels of these ingredients could have pronounced effects[13,14]. Thus, reduction of such materials in ECs does not assure total harm reduction. It is therefore important to evaluate the short-and-long-term safety of ECs on the cardiovascular system especially given the fact that there are controversial findings.

2.4.1 Acute EC Exposure

Early research on acute changes in CV vital signs (changes to blood pressure or heart rate (HR)) and vascular function were studied, but no consistent changes due to exposure were reported[16]. Farsalinos explains, any changes observed may be due to differences in exposure protocols, e-liquid nicotine concentrations and hardware designs. Others also reported no immediate effects to acute e-cigarette exposure on coronary circulation, myocardial infarction, and arterial stiffness[12]. Qasim et al. suggest that many discrepancies in their results could be due to inconsideration of vaping topography and small sample sizes which make it difficult to draw conclusions between CV health and EC exposure. Some studies have proposed the opposite[79], detecting increased heart rate[79,80] and blood pressure[81] in users. Vansickel et al.[79] demonstrated negative impacts to heart rate after EC use, while Yan et al.[80] detected elevated diastolic blood

pressure in conjunction with increased heart rate. However, both CV vital signs measured were lower when compared to TC use.

2.4.2 Chronic EC Exposure

As ECs have only been in the market for a short time relative to tobacco, long-term effects of ECs are relatively unknown[13]. Regardless, a growing body of studies have begun to examine the use of EC in human subjects[14,82]. In particular, a cross-sectional study[82] examining daily EC use showed an increase in myocardial infarction (OR = 1.79) but, this was less than that associated with chronic TC smoking (OR = 2.72). Of course, the conclusions drawn in this study are limited due to the fact that cross-sectional data does not establish a temporal relationship. Therefore, it is unknown whether EC users actually suffered myocardial infarctions while smoking TCs prior to switching as a cessation aid.

2.5 Assessing CV Health

Since long-term population data are unavailable and recent studies so far have resulted in controversial findings, we must also consider indirect ways of assessing cardiovascular health from available data on TC- induced CVD. As such, biomarkers like autonomic dysregulation are mechanisms predictive of CVD in TC smokers. This therefore may be useful in determining abnormalities from EC use.

The CV system displays characteristics typical of self-organization designed to maintain stability or homeostasis. This is achieved by the autonomically mediated control of heart rate, blood pressure, and other factors that react rapidly to metabolic stimuli via nerve fibers located throughout the body[83]. As such, within the autonomic nervous system (ANS), we distinguish two main components: the sympathetic (SS) and parasympathetic systems (PS). The SS uses

acetylcholine and norepinephrine as neurotransmitters. During stressful situations, they are released and in particular, norepinephrine is metabolized slowly in the blood stream[84,85]. This slow response enables changes in cardiovascular function to increase heart rate.

On the other hand, the PS uses acetylcholine as a neurotransmitter. Acetylcholine possesses a very short latency period and fast decay, thereby slowing heart rate. Both branches act in tandem with one another, therefore greater or lesser activation of both systems and differences in response times cause continuous changes in heart rate[85]. The ANS can also be stimulated mechanically. For example, arterial baroreceptors (afferent nerve fibers located in the aortic arch and carotid sinus) send inhibitory signals back to the brain that decreases central sympathetic nerve outflow and increase vagal outflow when increases in blood pressure arise[86].

Overall, changes in autonomic regulation of the heart have major implications in the cardiovascular system associated with arrhythmias and acute ischemia[3,13]. In fact, autonomic dysregulation also plays a role in the activation and progression of inflammatory atherosclerosis via production of proinflammatory cytokines and leukocyte mobilization. As norepinephrine is released from sympathetic nerve endings, it binds to α- or β- adrenergic receptors expressed on immune cells giving rise to proinflammatory cytokines and leukocyte recruitment[87]. The SS branch can also increase adhesion of leukocytes to endothelial cells in which activation and transendothelial migration can lead to foam cell and plaque formation[88]. In addition, influence on peripherally secreted proinflammatory cytokines like interleukin-1 and 6, and tumor necrosis factor- α can signal the brain via afferent fibers leading to SS activation[87].

2.5.1 HRV as an Indicator for CV Health

In general, acute and long-term exposure to cigarette smoke has been shown to lead to acute and chronic changes in the balance of the ANS resulting in sympathetic dominance via the

use of investigative techniques such as heart rate variability[13,89,90]. HRV was developed as a non-invasive diagnostic method used to asses autonomic health and determine CV prognosis.[86]. High variability implies good cardiovascular health and well-functioning autonomic control mechanisms. Low variability is thus indicative of bad CV health and associated with insufficient adaptability of the ANS to maintain homeostasis with respect to heart rate. In fact, it has been determined that exposure to TC smoke and PM promote CV pathogenesis via the dysregulation of the ANS leading to the release of catecholamines (e.g. norepinephrine) and resulting in increased sympathetic tone[15] as seen in some HRV studies[86,90].

In collaboration with the Araujo laboratory, Moheimani et al. determined whether this shift could occur in chronic EC users vs. age-matched, non-smoking controls[14]. They found abnormal HRV parameters in EC users consistent with increases in sympathetic activation, comparable to increased CV risk in patients with and without CVD . In addition, a follow up study[89] conducted by the same investigative team compared the effects of nicotine vs. non-nicotine. Acute increases in sympathetic activation assessed by HRV was detected with respect to the nicotine users. As such, the authors suggest that inhaled nicotine plays a substantial role in autonomic health. Based on these findings, the mechanisms by which elevated sympathetic nerve activity is observed could contribute to adverse cardiac events in EC users including arrhythmias and ischemia[3]. However, this study focused solely on frequency parameters with disregard to time domain parameters associated with HRV. Given the relationship between HRV and ANS any illness affecting the ANS can thereby affect HRV complicating conclusion drawn between pathologies[95]. Thus, further exploration into understanding EC- induced autonomic dysregulation by studying more than one HRV index is warranted.

2.6 Testing Models

In addition to mechanistic work there is the need for experimental models. Until recently, no reports of animal models exposed to EC were available resembling real-life exposure conditions like vaping topography. Nonetheless, certain studies have highlighted the usefulness of rodent models with respect to the nicotine pharmacokinetics from EC exposure [91], hepatotoxicity [92], and cardiac function in mice [93]. Shao et al. found that chronic EC exposure in Apolipoprotein E deficient mice (ApoE -/-) resulted in decreased body weight, food intake and increased locomotion. Furthermore, his rodent exposure system and chronic intermittent method yielded clinically relevant nicotine pharmacokinetics associated with behavioral and metabolic changes. Additionally, Hasan et al., detected significantly increased hepatic lipid accumulation in ApoE -/- mice with western diets and EC exposure (with 2.4% nicotine concentration) vs. ApoE -/- mice on similar diets and exposed to saline aerosol. Espinoza-Derout et al. demonstrated adverse cardiac function in ApoE null mice exposed to 2.4% nicotine in EC aerosol. Those exposed to e-cigarette emissions had increased atherosclerotic lesions, decreased left ventricular shortening and reduced ejection fraction.

All studies used a similar exposure model system specifically developed to test EC exposures in rodents taking into consideration human vaping topography. While extrapolation from animal models to humans should be handled cautiously, these investigations show the potential e-cigarette-specific murine models may have in understanding adverse health outcomes from ENDS. Given the paucity of data from long-term population studies, these rodent exposure systems may provide essential data faster with which policies and regulations may be informed. With no current mouse data available specific to HRV and EC exposure, this opens an avenue to further elucidate the human evidence detecting autonomic dysregulation from ECs.

3. MATERIALS AND METHODS

3.1 C57BL/6 Mice

Males with a C57BL/6 background were obtained from DLAM Breeding Colony Services, UCLA. Mice, age 12 weeks, were anesthetized (isoflurane inhalation) and implanted with radio telemetry devices (TA10ETA-F20; Data Sciences Intl., St. Paul, MN). Transmitter units were installed in the abdomen and leads placed adjacent to the heart in a Lead II arrangement by Dr. Maria Jordan[94]. Mice were individually housed and maintained in a temperature controlled (26°C) facility with a strict 12:12-hour light: dark cycle and given free access to food and water. Animal handling and experimentation were in accordance with the recommendation of the Animal Research Committee and were approved by the David Geffen School of Medicine Division of Laboratory Animal Medicine (Protocol #19-016).

3.2 Chamber Set up

The Mouse EC Exposure Resource (MECER) was set up inside the Physiology Core under the care of Dr. Kenneth Roos and the Department of Laboratory Animal Medicine (DLAM) at UCLA. This efficient EC aerosol generation exposure system for rodents was invented by Dr. Max Shao (Patent PCT/US1745133) and two were purchased (Automate Scientific. Inc.) for the purposes of this study (**Figure 2**). Each chamber is comprised of a pressure gauge, a solenoid valve, a multichannel valve, one manual valve, an EC holder, and an animal chamber wherein rodents were continually exposed to pressurized air with a flow rate of 5 L/min. The primary product used for exposure was the BluPLUS+ ™ rechargeable kit that included 1 battery and 1 USB charger per EC. In addition, Classic Tobacco flavoring with a 2.4% nicotine concentration was used[39].

Figure 2. E-cigarette Aerosol Generation Systems. Each chamber housed one C57BL/6 male mouse for the duration of the exposure.

3.3. Exposure Protocol

All experiments were performed in the conscious state between 8:00 AM to 3:00 PM. Mice (n = 6) were subjected to a "baseline" event where an individual animal was placed in a chamber for one hour to adjust for any increased HR when moving mice from their cages to chambers (**Figure 3**). This was followed by a 15-minute air (control) exposure followed by a 45-minute post-exposure with a flow rate of 5L/min at a pressure of 40 psi. Two EC exposures followed with a flow rate of 1 L/min and pressure of 10 psi, each with their own post-exposure period. Based on recommendations set forth by the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) e-cigarette Task Force, EC devices were activated for 4 seconds per puff, with an inter-puff delay of 26 seconds, every 30 seconds for the duration of 15 minutes. This totaled to 30 puffs per EC exposure. According to previous studies associated with MECER[91], aerosolized saline was used as a control, therefore we incorporated a secondary control measure using Dulbecco's 1X Phosphate- Buffered Saline (PBS). PBS was aerosolized via the use of a nebulizer and mice were exposed to this for 15 min for a flow rate of 4.5 L/min at a pressure of 40 psi. The duration of the protocol lasted for a total of five hours and mice were

returned to their individual cages after exposures. Recordings were conducted at each stage or event including resting periods with mice exposed once a week over a 6-week period.

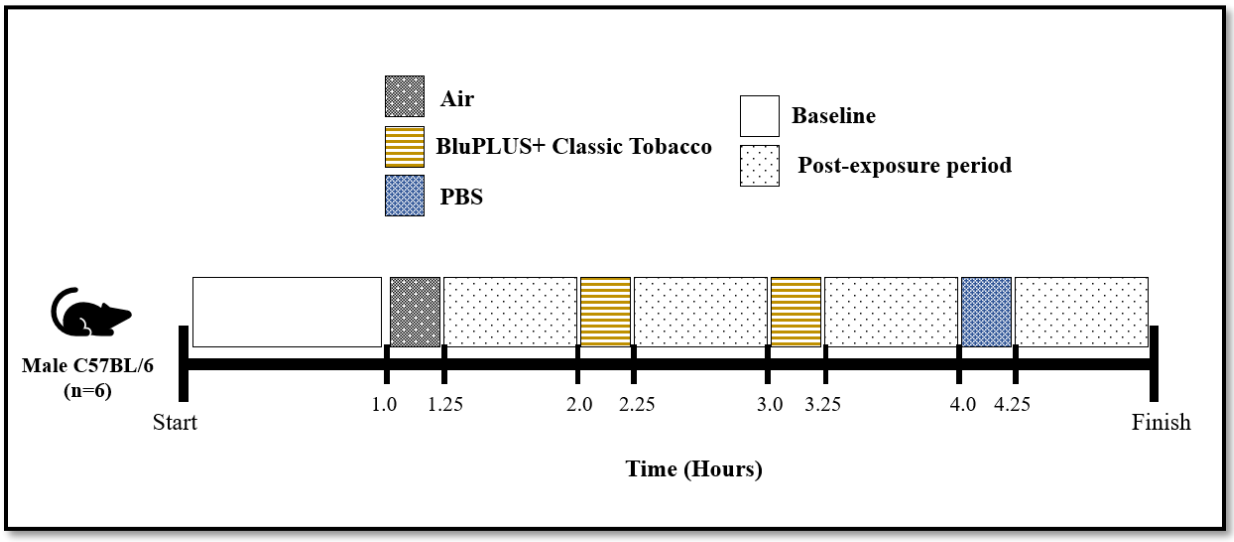

Figure 3. Schematic of Experimental Design.

3.3 Data Acquisition and HRV Analysis

Data recordings began six weeks after implantation (recovery time) via the use of an antenna receiver placed under the cage connected to a computer system located in a separate room within the Mouse Physiology Core. Echocardiograms (ECG) waveforms (P-R, QRS, Q-T), temperature, and activity were collected, analyzed and displayed with DSI Ponemah telemetry software (DSI^{TM}, Harvard Bioscience Inc., St. Paul, MN, USA) on a continuous basis for the duration of each study. All studies were performed in a dedicated telemetry room ensuring a quiet and undisturbed environment for the mice used in these experiments. Parameters were then analyzed with the DSI Ponemah program (Version 6.2) before and after EC and control exposures according to standard criteria[95].

It is essential that HRV analysis be performed on sinus beats only. The use of a graphic interface of the software program allowed for visual reviewing and manual removal of ectopic beats, sinus pauses, artifact, and noise. Therefore, for standardization only stable sinus rhythms were used for analysis. HRV was quantified using standard time domain and frequency domain techniques on the basis of recommendations[95,96].

3.3.1 Time Domain Measures

Time domain quantifies variability between beat to beat data obtained from RR interval series. Numerical indices summarizing variability of the series are calculated from the RR intervals. These measurements, after editing to remove abnormal beats (i.e., normalized) via the use of Ponemah, are then subjected to simple statistical analysis[90]. Normal RR intervals were quantified by calculating the mean RR interval and any RR series two standard deviations away were removed. Therefore, the time domain parameters reported include: the standard deviation of all normal RR intervals (SDNN, in milliseconds (ms)), the root means square difference between successive RR intervals (RMSSD, in ms), and the proportion of normal consecutive RR intervals differing by x (pNNx where x = 6 ms). The reporting period was set to 5-minute intervals following previously published recommendations[86,90,96] to examine the acute effects of EC exposure on HRV in mice.

3.3.2 Frequency Domain Measures

Because the sympathetic and parasympathetic branches of the ANS strongly influence heart rate, there is a difference in the speed by which heart rate changes occur. The sympathetic system is slow in its effects, while the parasympathetic is faster[90]. Given the difference of speed responses, frequency analysis can be used to study sympathetic and parasympathetic contributions to HRV. Data was interpolated at 50 Hertz (Hz) using a quadratic method and

tolerance set at 20 ms. Furthermore, data was detrended and multiplied by a Hanning window with overlapping of sub-series set to 2 to meet the requirements for performing a discrete Fast Fourier Transform (DFT). The squared magnitudes of the DFT were then averaged to form a power spectral density of the beat interval time series- computed by use of a modified averaged periodogram set to max in the DSI Ponemah program (Version 6.2).

Segment durations were set to one- minute intervals and all data was parsed with respect to duration of exposures not accounting for bad error marks, artifact and signal noise. Frequency domain parameters reported include low frequency (LF in ms^2), high frequency (HF in ms^2), and the ratio between LF and HF (LF/HF). Cut off frequencies for power in the LF range were set to 0.40 - 1.5 Hz, while HF limits were set to 1.5Hz - 4 Hz in accordance to previously published recommendations[96].

3.4 Aerosol Characterization

To understand what kinds of particles mice are exposed to in the chambers, particle number, particle mass concentration, and particle size distributions were measured in real time in collaboration with Dr. Yifang Zhu. Condensation Particle Counter (CPC) Model 3007 ((TSI Inc., Shoreview, MN, USA) and DustTrak II Aerosol Monitor 8532 (TSI Inc., Shoreview, MN, USA) were used to measure real-time Particle Number Concentration (PNC) and Particle Mass Concentrations (PMC) at 1 second intervals. Particle size distributions were measured by a Scanning Mobility Particle Sizer (SMPS) 3080 (TSI Inc., Shoreview, MN, USA) (0.6 L/minute sampling flow rate; 100-s up scan, 20-s down scan) and an Aerodynamic Particle Sizer (APS) 3321 (TSI Inc., Shoreview, MN, USA)[97] in the size ranges of 7.39 - 289 nm and 0.5 - 19.8 µm, respectively. TSI Data Merge Software Module (TSI Inc., Shoreview, MN), which converts

electronic mobility diameter measured by the SMPS to aerodynamic diameter was used to merge SMPS data with APS resulting in composite size distributions.

In addition, a personal cascade impactor (Sioutas Cascade Impactor, SKC Inc., Eighty-Four, PA, USA) was used to measure $PM_{2.5}$ mass concentration during a 15-min EC exposure period following the gravimetric method[98]. The personal cascade impactor was used to provide a cut-size of 2.5µm and collect particles less than 2.5 µm on a 37mm Teflon membrane filter (SKC Inc.). The sampling flow rate was 9.2 L/ min. Instruments were directly connected to one exposure chamber without mice present. Each exposure (Air, EC and PBS) was characterized for 15 min followed by their respective resting periods (45 min).

3.5 Statistical Analysis

Aerosol distribution data was plotted using normalized concentrations ($dN/dlogD_p$) to correct for differences between instruments and provide a best fit for single source aerosols. With no real differences, air and post air exposures were combined (combined air) and physiological data were reported in means ± standard error of the mean (SEM). HRV data gathered from Ponemah was screened to exclude values deemed implausible or impossible for the following HRV parameters: SDNN > 1000, RMSSD > 1000, NN > 300, LF > 1000, HF > 1000, LF/HF > 20. All pNNx data points were included in the analysis. For each HRV parameter, a linear mixed-effects regression analysis was carried out to estimate the mean HRV in the different experimental conditions.

The outcome variables LF and HF were entered into the regression analysis log-transformed in order to promote normal distribution of the residuals. Each regression model

included the categorical predictors Trial (levels: 1, 2, 3) and Event (levels: Air, EC1, Post-EC1, EC2, Post-EC2, PBS, Post-PBS), and a random subject-level intercept to account for between-subjects heterogeneity. A robust estimation method was used to obtain parameter estimates that are robust to the presence of extreme values and outliers. For further analysis, custom contrasts were used to test specific hypotheses about differences between events or combinations of events. The alpha level was set at 0.05. All analyses were performed with the statistical software R (Version 3.6.3) and in consultation with Dennis Rünger, a senior statistician in the Department of Medicine Statistics Core.

4. RESULTS

4.1 Aerosol Characterization

Combined real-time measurements by SMPS and APS instruments of EC and PBS during their respective 15-minute exposures followed by its 45-minute post-exposure event resulted in similar patterns, albeit different concentrations (**Figure 4A-B**). Over a 15- minute exposure period, EC aerosol remained consistent at an overall particle number concentration of approximately 3.0×10^6 #/cm^3; particle mass concentration was ~2.0×10^4 µg/m^3. Number and mass concentrations of PBS aerosol were lower, however during exposure, aerosol generation was consistent (5.24×10^5 #/ cm^3; 1.75×10^3 µg/m^3). This quickly dropped during the post-exposure phase within a 5-minute time span (1.90×10^5 #/cm^3; 562 µg/m^3). In contrast, EC aerosol decreased within a 20 to 25-minute period (200 #/cm^3; 0.4 µg/m^3). Air remained consistent with a mean particle number concentration of 1.58×10^3 #/ cm^3 and particle mass concentration of 1.61 µg/m^3.

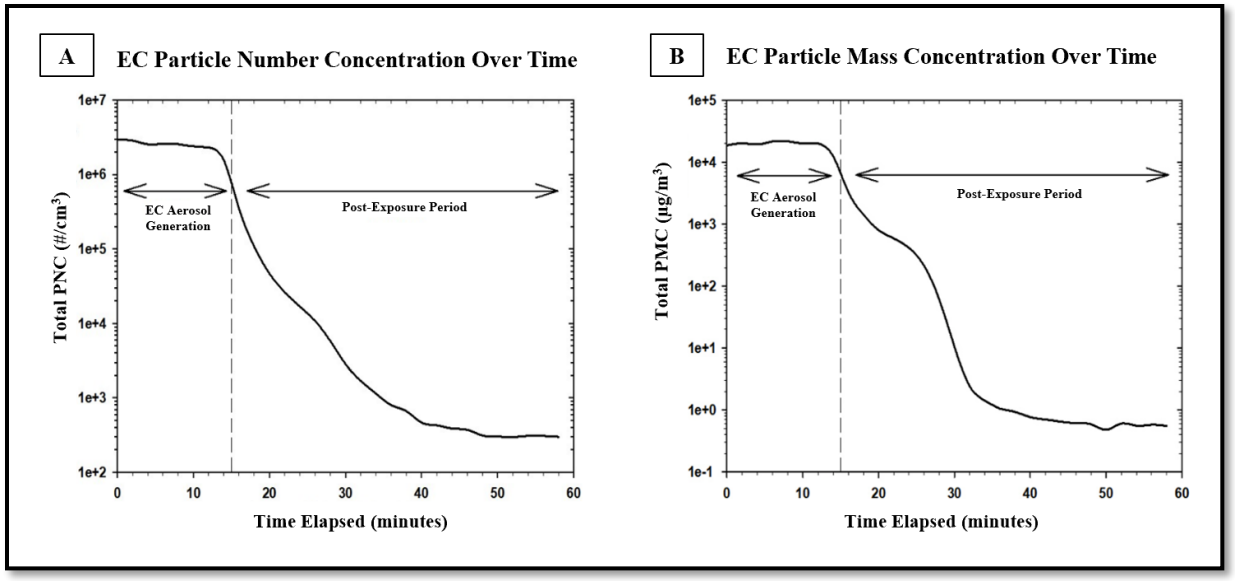

Figure 4. Time Series Plots of Total EC Particle Number and Mass Concentrations. EC particle number (4A) and particle mass (4B) values were obtained from the exposure chamber during a 15-minute aerosol generation followed by 45-minute post-exposure period for a total of 60-minutes.

The averaged particle size distribution of seven measurements completed during the 15-minute EC, PBS and air exposures are shown in **Figure 5**. Normalized PNC (**Figure 5A**) demonstrated a greater bimodal distribution of EC aerosol at approximately 0.29 µm (3×10^7 #/cm^3) and 0.9 µm (4×10^4 #/cm^3). At 0.5 µm, EC PNC dropped by three orders of magnitude and as particle size increased, particle number distributions decreased with no particles measured at a 0.4 µm size. PBS PNC was multi-modal and was an order of magnitude smaller at its highest particle size of 0.045µm (**Figure 5B**), while air's highest number concentration was at 3×10^3 #/cm^3 with a particle size of 0.07 µm **(Figure 5C**). Normalized particle mass showed similar results; EC mass concentrations peaked at 3.0×10^5 µg/m^3 in particles with a size of approximately 0.29 µm and at 2.5 µm (5×10^3 µg/m^3) (**Figure 5D**), while PBS normalized mass concentrations were two orders of magnitude (1.0×10^3 µg/m^3) lower than EC (**Figure 5E**). However, PBS mass distributions were more variable than EC curves, similar to the trend seen in its number distribution. Overall, PBS data suggest lower number and mass concentrations as

compared to EC. Air measured resulted in levels lower to both EC and PBS (**Figure 5E-F**). In addition, the average $PM_{2.5}$ mass concentration during a 15-min exposure period of EC was measured at 8.1 mg/m^3 (8,072 µg/m^3).

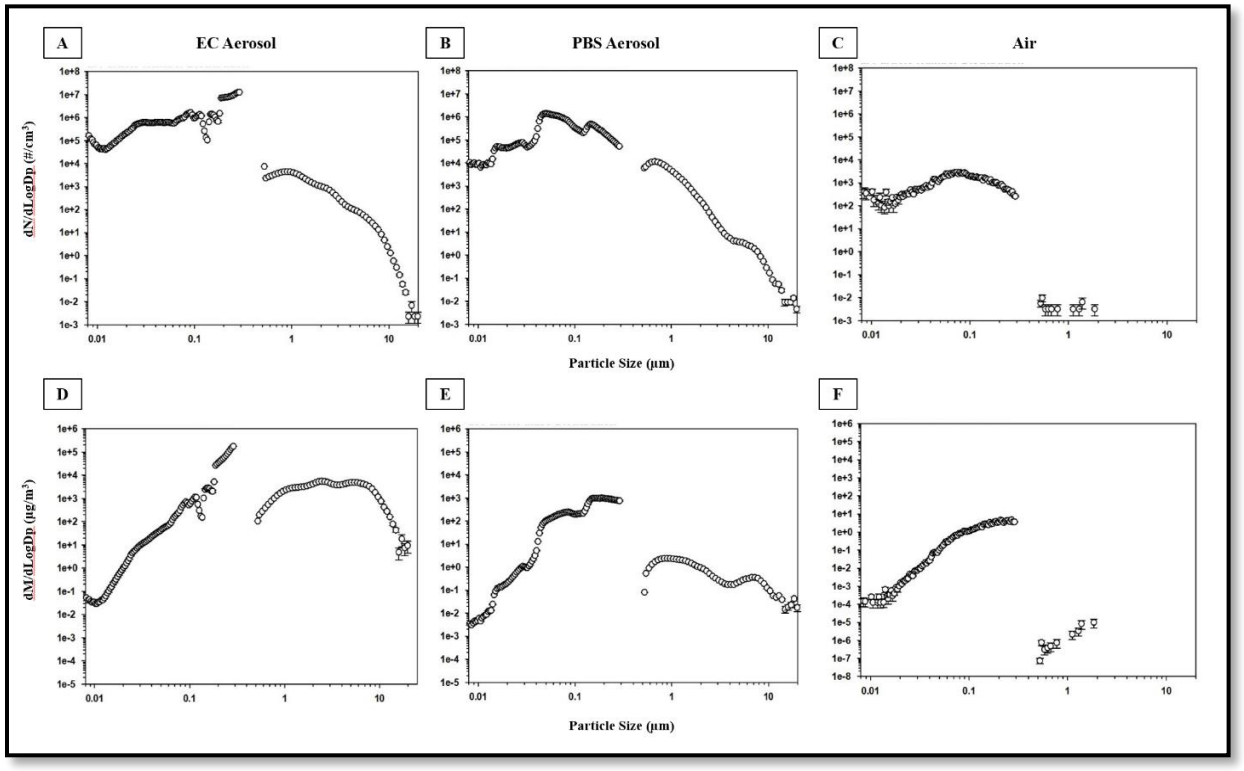

Figure 5. Normalized Particle Number and Mass Distributions. SMPS and APS data were merged and normalized using dN/dLogDp for particle number and mass distributions. The dots represent the mean dN/dLogDp for EC aerosol (A), PBS (B), and Air (C) for particle number distribution. For particle mass distribution, dots represent dM/dLogDp values for EC (D), PBS (E), and Air (F). 7 measurements were taken in the 15-minute period and the width of the error bar represents one standard error of these measurements.

4.2 Physiological Characteristics

For a normal cardiac cycle, the rhythmic contraction and relaxation of the heart may provide valuable insight into cardiac abnormalities. As the ability to initiate electrical impulses spontaneously can be influenced by autonomic nerves, ECG parameters: QRS complex, ST segment, QT interval, and for each exposure period of all six mice were analyzed and reported in **Table 1**. The QRS complex values, indicating depolarization of the ventricles, fell under normal

ranges with little change between exposures. Mean values of ST segment reflecting repolarization of the myocardium, demonstrate reductions in EC 1 (101.51 ± 2.33) and EC 2 (97.15 ± 2.60) exposures as compared to the combined air event (116.25 ± 2.03). However, this slightly increases during the PBS period (121.99 ± 2.98). QT interval values, the time duration between onset of the QRS complex to the end of the T wave, reflect shorter durations in both EC exposures, while post-exposures indicate values comparable to the combined air event (129.34 ± 1.94). PBS slightly increases the QT interval (132.59 ± 2.98), indicating a longer time span.

Table 1. Physiological Characteristics of C57BL/6 mice.

Exposures	QRS† (ms)	ST† (ms)	QT† (ms)	HR† (bpm)	T† (°C)
Baseline	13.11 (0.028)	124.84 (1.50)	135.76 (1.49)	638.30 (2.08)	38.10 (0.019)
Air	13.54 (0.166)	116.25 (2.03)	129.34 (1.94)	578.14 (2.43)	37.11 (0.030)
EC 1	13.37 (0.045)	101.51 (2.33)	111.92 (2.33)	565.84 (5.09)	36.71 (0.054)
Post-EC 1	13.45 (0.039)	118.17 (1.81)	128.53 (1.80)	599.55 (3.10)	36.88 (0.038)
EC 2	13.59 (0.053)	97.15 (2.60)	107.34 (2.58)	543.44 (5.44)	36.41 (0.066)
Post-EC 2	13.55 (0.030)	114.99 (2.35)	125.53 (2.36)	588.87 (3.26)	36.25 (0.045)
PBS	13.60 (0.073)	121.99 (2.98)	132.59 (2.98)	598.87 (5.18)	36.64 (0.061)
Post-PBS	13.78 (0.034)	110.85 (1.98)	121.49 (1.97)	533.58 (3.80)	35.85 (0.043)

†Values denote the mean of each ECG parameter for all mice (n = 6) exposed over three trials with SEM in parentheses. Total data points are shown in parentheses under each ECG parameter.

Heart rate (beats per minute (bpm)) and Temperature (T) in degrees Celsius were also reported. Mice bodily temperatures are within normal range varying between ~36.5- 39 °C (**Table 1**). HR is at its highest during baseline (638.30 ± 2.08) yet, decreases during both EC events in comparison to the combined air exposure (578.14 ± 2.14). Post-EC events elevate HR, higher than the control, however post-PBS induces the lowest levels of reductions in HR (533.58 ± 3.80). Similar to trends observed in the QT interval, PBS largely increases heart rate (598.87 ± 5.18) compared to combined air. Overall, SEMs in the exposure period suggests more variation within the data set as opposed to the control event.

4.3 Time Domain Analysis

HRV is commonly assessed by analyzing the time domain which represents the total amount of variability and is influenced by changes in both sympathetic and parasympathetic activity. Thus, SDNN, an indicator of total autonomic variability, and RMSSD, a beat to beat index of HRV reflecting short-term variations in heart rate, were analyzed to assess whether changes in HRV occurred in the experimental design. When compared to baseline (Figure 6A-B), a significant drop was observed following combined air. Similarly, EC 1 (-3.63, $CI_{95\%}$ [-8.43 - 1.18]; -6.15, $CI_{95\%}$ [-13.37, 1.06]) and EC 2 (-6.38, $CI_{95\%}$ [-11.29, -1.47]; -12.52, $CI_{95\%}$ [-19.89, -5.16]) show reductions in SDNN and RMSSD, respectively. Significant increases in post-EC 1 (5.86, $CI_{95\%}$ [2.46, 9.27]; 9.36, $CI_{95\%}$ [4.25, 14.46]) and post-EC 2 (-4.67, $CI_{95\%}$ [-8.05, -1.28]; -8.81, $CI_{95\%}$ [-13.90, -3.72]) suggest these exposures differ when compared to combined air. Reductions during the post-PBS event was also significant in the RMSSD (-7.55, $CI_{95\%}$ [-12.66, -2.45]).

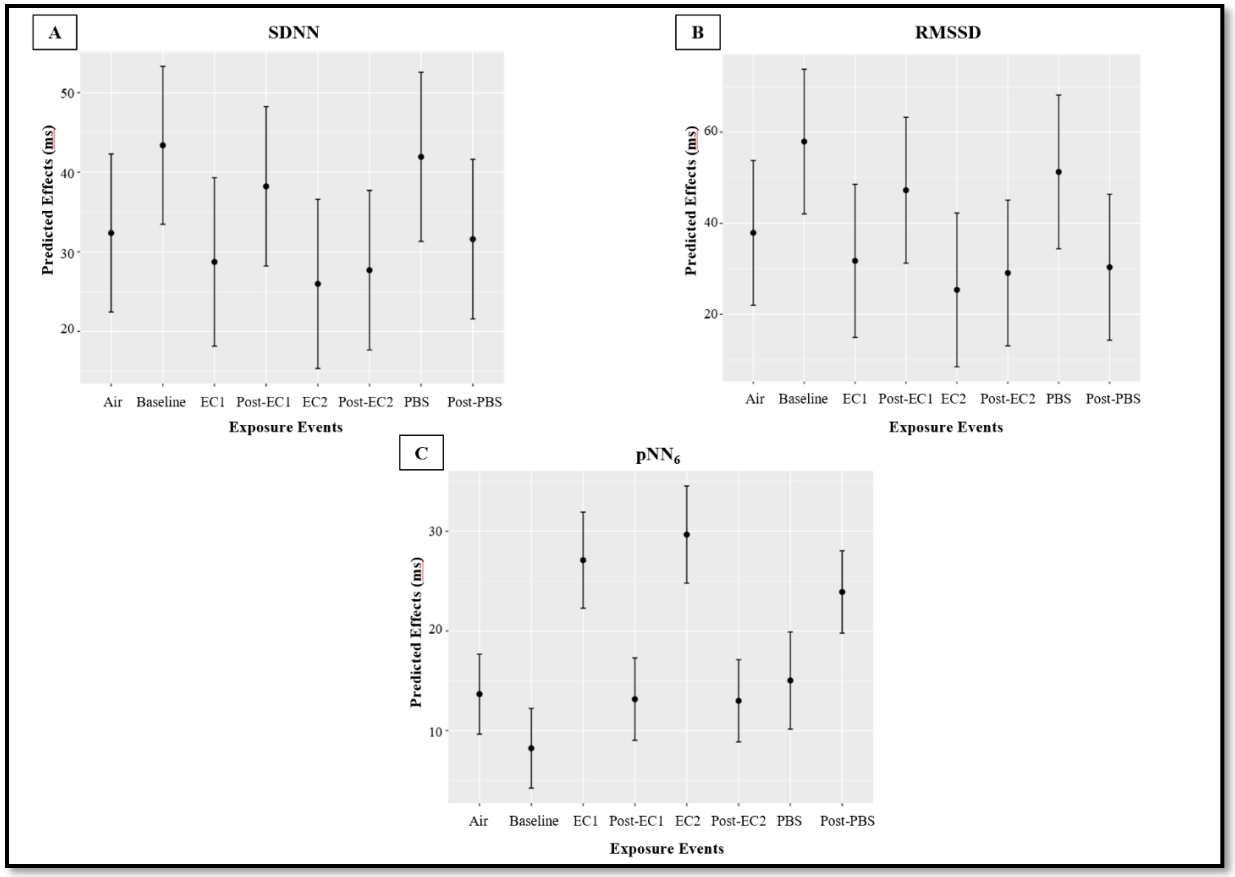

Figure 6. HRV Reductions Measured During EC Exposure in Time Domain Parameters. Black dots denote the mean for each event, whereas vertical lines represent lower and upper limits or the 95% confidence intervals. Events were compared to the control event, Air, and plotted using R to predict how time domain parameters will change when all other events or covariates are kept fixed. Figure 6A-B shows reductions in RMSSD and SDNN when mice were exposed to EC with a larger reduction during the EC 2 than EC 1. However, pNN6 (Figure 6C) denotes an increase in both EC exposures and overall opposite trends in each event in comparison to SDNN and RMSSD.

Time domain parameter, pNN_6, an indicator of short-term variability similar to RMSSD, shows the opposite trends (**Figure 6C**). When compared to air, baseline (-5.43, $CI_{95\%}$ [-7.67, -3.19]), post-EC 1 (-0.51, $CI_{95\%}$ [9.93, 16.94]) and post- EC 2 (-0.67, $CI_{95\%}$ [-3.14, 1.81]) decreased, however only baseline was significant. While EC 1, EC 2, and post-PBS exposures significantly differed from air by 13.44, 16.00, and 10.24 units, respectively, PBS was slightly elevated as compared to combined air by 1.37 ($CI_{95\%}$ [-2.21, 4.95]).

To further explore whether EC aerosol could induce effects in HRV, key contrasts and outcomes were evaluated (**Table 2**). As time domain data is aggregated by 5-minute intervals,

EC exposure events and post-EC events were combined, independently. When comparing pooled EC and combined air, EC exposures significantly decreased SDNN and RMSSD by 5.00 ($CI_{95\%}$ [-8.28, -1.23]) and 9.34 ($CI_{95\%}$ [-15.01, -3.67] units, respectively. In conjunction, EC vs. PBS resulted in greater reductions in both parameters (-14.58, $CI_{95\%}$ [-19.92, -9.24] in SDNN; -22.72, $CI_{95\%}$ (30.74, -14.71] in RMSSD). Both parameters increase in the post-EC periods as compared with EC exposures. Similarly, PBS exposures elevated SDNN and RMSSD in contrast to combined air. Conversely, these results were inconsistent with pNN_6 values as a higher proportion of adjacent interbeat intervals differed by more than 6ms in the EC events when compared to combined air (14.72, $CI_{95\%}$ [11.96, 17.47]). Similar trends were observed in EC vs. PBS and post-EC.

Table 2. Custom Contrasts for Time Domain Parameters. RMSSD and SDNN, measures of non-specific autonomic variability, are decreased during EC events compared to combined air (control) and PBS (secondary control). Significant differences were also measured between controls and between EC and post-EC events. In contrast, significant increases in pNN_6 suggest increases in HRV.

Exposure Comparisons	SDNN† (n = 1109)	RMSSD† (n = 1109)	pNN_6† (n = 1113)
EC vs. Air	-5.00** (1.93)	-9.34*** (2.89)	14.72**** (1.40)
EC vs. PBS	-14.58**** (2.72)	-22.72**** (4.09)	13.35**** (1.99)
EC vs. Post-EC	-5.60** (1.82)	-9.61*** (2.73)	15.30**** (1.32)
PBS vs. Air	9.57**** (2.50)	13.38**** (3.76)	1.37 (1.83)

†Values indicate regression coefficients with standard error shown in parentheses as determined by a linear mixed model with a robust estimator. **, *, **** indicate significance at the 99%, 99.9% and <99.9% level, respectively.**

4.4 Frequency Domain Analysis

Log-transformed frequency domain components LF, a measure of sympathetic activity, HF, an indicator of parasympathetic activity, and LF/HF, a ratio reflecting sympathovagal balance were analyzed to determine direct physiological changes of HRV. All EC exposure events were significantly higher than combined air (2.29, $CI_{95\%}$ [1.50, 3.07]), however, baseline and post-events were lower than EC 1 (1.25, $CI_{95\%}$ [1.01, 1.49]), EC 2 (1.41, $CI_{95\%}$ [1.17, 1.64]) and PBS (1.10, $CI_{95\%}$ [0.78, 1.41]) events under the LF component (**Figure 7A**). A similar trend continued in **Figure 7B**, with increases in all exposure events when compared to combined air. However, in contrast to LF, values in the HF parameter were an order of magnitude lower with increases in baseline (1.34, $CI_{95\%}$ [1.13, 1.55]) and PBS (1.28, $CI_{95\%}$ [0.96, 1.61]). Comparably, incremental results in log (LF/HF) during the baseline and PBS events are also observed in comparison to combined air (3.26, $CI_{95\%}$ [3.16, 4.09]). In contrast, EC 1 was significantly reduced with more than a two-fold difference when logarithmically compared to combined air (-0.38, $CI_{95\%}$ [-0.74, -0.01]) while EC 2 (0.16, $CI_{95\%}$ [-0.20, 0.53]) was slightly elevated. Post-EC events were higher than air suggesting a shift in sympathovagal balance towards sympathetic predominance (**Figure 7C**).

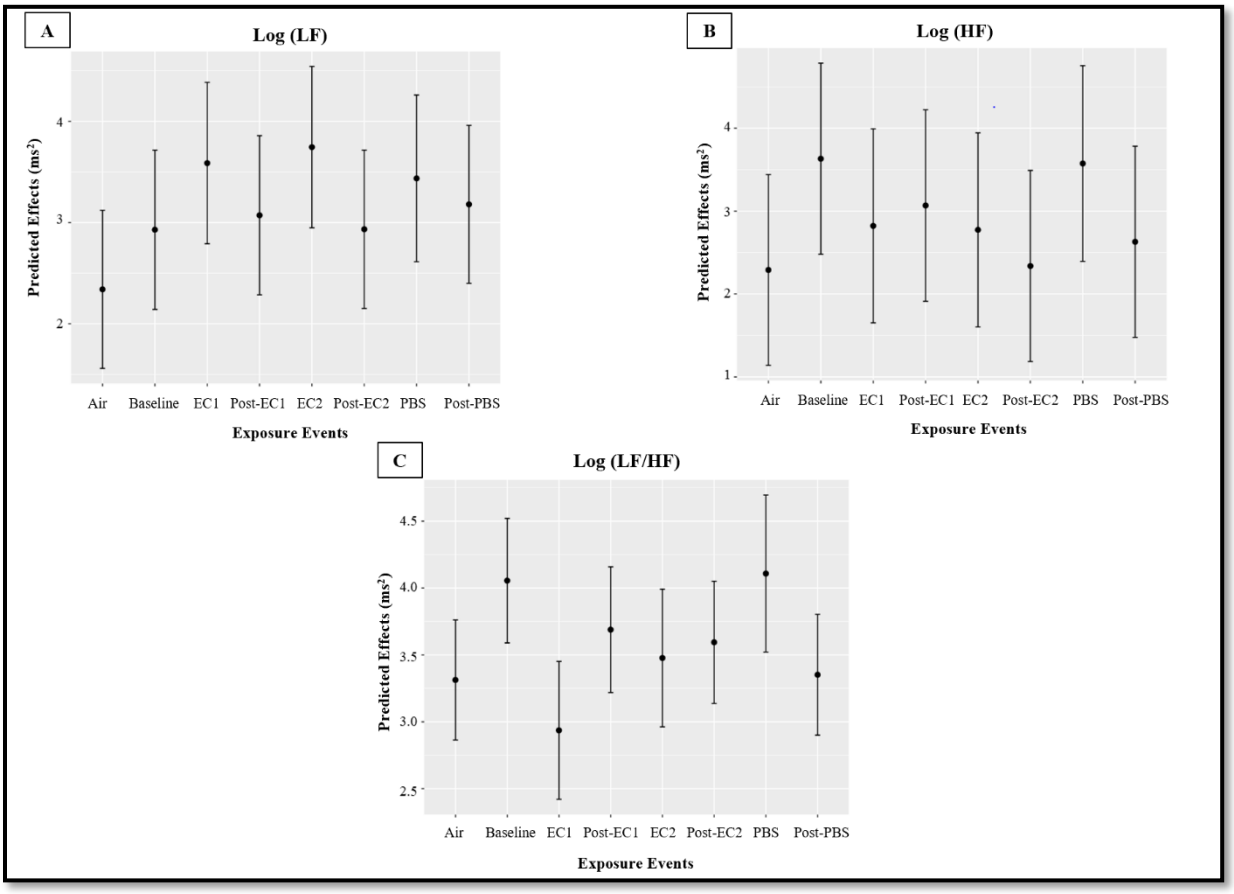

Figure 7. Marginal Effects of Log- transformed Frequency Domain Parameters. Black dots denote the log (mean) for each event, whereas vertical lines represent lower and upper limits or the 95% confidence intervals. Events were compared to the control event, Air, and plotted using R to predict how frequency domain parameters would change when all other events or covariates were kept fixed. Figures 7A-B show elevations in log (LF) and log (HF), with reductions in the LF/HF ratio (7C) during EC exposures as compared to air and PBS.

In **Table 3**, EC exposure significantly increased in the LF component by 1.33 units ($CI_{95\%}$ [1.14, 1.52]), suggesting this combined exposure event (geometric mean = 37.34) is 3.78 times higher than combined air (geometric mean = 9.87). Significant elevations in HF were also observed, however estimates were lower (0.51; $CI_{95\%}$ [0.28, 0.73]) than LF. Conversely, LF/HF estimates indicated a decrease of less than an order of magnitude (-0.11) during EC exposures, yet this trend is not significant ($CI_{95\%}$ [-0.40, 0.19]). During PBS exposures, LF increased (0.23) in the EC event; while not significant ($CI_{95\%}$ [-0.09, .55]), this pattern is consistent with EC vs.

air results. In contrast, HF (-0.78, $CI_{95\%}$ [-1.12, -0.43]) and LF/HF (-0.90, $CI_{95\%}$ [-1.38, -0.42]) drastically decreased during the EC period.

Table 3. Custom Contrasts for Frequency Domain Parameters. Increased log (LF), a measure suggestive of sympathetic activity, was measure during EC events when compared to the primary (air) and secondary (PBS) controls. Log (HF), an indicator of vagal activity, was also significantly elevated, however, the magnitude is less than that of log (LF). Conversely, LF/HF was reduced when compared to the controls vs. pooled EC events suggesting parasympathetic dominance.

Exposure Comparisons	LF† (n = 3057)	HF† (n = 3850)	LF/HF† (n = 3250)
EC vs. Air	1.33**** (0.10)	0.51**** (0.12)	-0.11 (0.15)
EC vs. PBS	0.23 (0.17)	-0.78**** (4.09)	-0.90**** (0.24)
EC vs. Post-EC	0.66**** (0.09)	0.10 (0.11)	-0.43*** (0.14)
PBS vs. Air	1.10**** (0.16)	1.28**** (0.17)	0.80*** (0.23)

†Values indicate regression coefficients with standard error shown in parentheses as determined by a linear mixed model with a robust estimator. *, **** indicate significance at the 99.9% and 99.99% level, respectively.**

Collectively, LF and HF results suggest sympathetic dominance consistent with reductions in the SDNN and RMSSD however, reductions of LF/HF indicate a shift in sympathovagal balance towards parasympathetic activity. Comparisons between EC vs. post-EC exposures exhibit differences in the LF (0.66, $CI_{95\%}$ [0.48, 0.84]), HF (0.10, $CI_{95\%}$ [-0.12, 0.31]), and LF/HF ratio (-0.43, $CI_{95\%}$ [-0.71, -0.16]). Contrasts between PBS and combined air showed significant increases of all frequency domain parameters with a slightly higher elevation in the HF parameter.

5. DISCUSSION

5.1 Experimental Design and Vaping Topography Considerations

We aimed to develop a sensitive *in-vivo* exposure model with an experimental design that resembled real-life scenarios for acute exposure due to serious methodological problems in acute CV human studies and until recently, a lack of exposure models mimicking vaping topography. Previous MECER models were primarily introduced as nicotine aerosol delivery systems for chronic and acute intermittent exposures. In developing this system, we needed to adapt this model for acute e-cigarette exposures with the addition of telemetry devices. To reproduce human vaping topography in mice, an inhalation route of administration was assessed lasting 15-minutes with a 4 second puff every 30 seconds. 30 puffs per exposure were comparable to previous human studies[46,99]. As with TCs, EC episodic inhalation patterns induce activation, de-sensitization, and re-sensitization cycles important in nicotine pharmacokinetics[91]. Therefore, to imitate the episodic nature of vaping seen in EC users two exposures were introduced into the experimental design with intermittent events lasting 45-minutes. Temporal profiles demonstrated that number and mass concentrations remained consistent within the aerosol generation stage and dropped in both parameters during the post-exposure period. This indicated a 45-minute time span was enough to reduce EC aerosols comparable to air as indicated by previous studies[91-93].

Aerosol generation factors such as puff duration, interpuff intervals, and intermittent exposures can also impact particle characteristics in addition to human topography[38]. Therefore, flow rate (1 L/min during EC; 5 L/min in all other events) and vapor pressure (40 psi during EC; 10 psi in all other events) were fixed to ensure consistency across different experiments. Notably, the observed EC particle number concentrations were comparable to those reported by Mikheev

et al.[100] and exhibited a bimodal particle size distribution with larger concentrations in the fine and ultrafine range similar to previous reports[101-103]. However, recent studies have reported PNCs of 10^9 #/cm^3 in ECs and conventional cigarettes[103,104], suggesting that values obtained in our study are lower, possibly due to a higher dilution ratio of the flow rate favoring evaporation rather than coagulation of particles. On the other hand, coagulation can also lead to a reduction in particle number concentration as seen in these results, especially with longer puff duration and small chamber sizes[38]. Additionally, particle mass concentrations demonstrate similar distributions and particle size ranges. This is further supported by the observed gradual decrease of EC particles after aerosol generation supporting the idea that there is a rapid loss of EC aerosol particles. Interestingly, this process is similar to TC particle generation[38].

In particular, a recent report by Lampos and colleagues suggest rapid vaporization of EC ultrafine particles ($PM_{0.1}$) are attributable to volatile material, possibly PG or GLY[103]. Lampos et al. reported a 10 to 20-sec evaporation in both number and mass in contrast to the 20-minutes for EC aerosols to decrease in our chambers. This may be explained by the presence of fine particles thought to be generated by pyrolysis, metals, and low-volatile chemicals[13,100] which do not evaporate quickly. However, environmental factors such as temperature and relative humidity were not measured and may have impacted PNC, PMC and particle size distributions. Further physical characterization and chemical composition analysis will be required to include these factors in relation to EC aerosol.

Interestingly, PBS demonstrated a similar particle number and mass distribution to EC albeit an order of magnitude lower. While EC particles are mainly generated by pyrolysis, PBS is not heated and are composed of salts. Additionally, PNC and PMC are several orders of magnitude lower than PBS in comparison to air which may be attributable to PBS' non-toxic

isotonic salt solution. Unfortunately, no data on aerosolized PBS characterization is available given that it is mainly used for the handling and culturing of mammalian cells[105]. While this study is not the first of its kind to use aerosolized PBS as a control[106,107] on animal subjects, further physical characterization should be assessed to better understand its aerosol dynamics.

5.2 PM and Potential Health Impacts

EC $PM_{2.5}$ concentrations were measured using a personal cascade impactor attached to one exposure chamber over a 15-minute period. In various studies[103] mean concentration of $PM_{2.5}$ varied between 0.5 to 197 µg/m^3. However, Volesksy et al. reported a higher mean range between 394 – 1,117 µg/m^3 (for cigalikes)[103]. In contrast, mean concentration obtained by this study was 6.8-fold to 20-fold higher. E-cigarette aerosol measured, room and chamber size, indoor climate, and airflow factors likely influence this drastic change. Regardless, this level surpasses not only these reports but also the U.S. Environmental Protection Agency (EPA) standard of 35 µg/m^3. Of course, these guidelines are for 24-hour averages, decreasing its comparability, but not its importance as secondhand-exposures with environmental and biological implications.

In particular, it is recommended that PM be kept as low as possible as clinical studies have demonstrated an association between ambient PM and increased risk of death from CVD [108,109]. According to the World Health Organization (WHO), $PM_{2.5}$ safety level guidelines are set at 25 µg/m^3 in which case, PM levels detected in this study far exceed that standard. However, ambient $PM_{2.5}$ concentrations have a completely different chemical composition to ECs. Fine particles are produced through the combustion of fossil fuels in which organic and metal components vary by location[108]. Common elements of ambient PM include nitrates, PAHs,

metals, sulfates, and organic and elemental carbon in which particle formation is dependent on other pollutants and atmospheric conditions[108-110]. ECs on the other hand, are composed of PG, GLY, nicotine, and flavorings where fine particles are produced via pyrolysis. Hence, we cannot assume $PM_{2.5}$ from ECs will have the same biological toxicity as ambient $PM_{2.5}$. Moreover, its mere presence from ECs does not mean EC- derived $PM_{2.5}$ will have an effect. However, chemical composition of the e-liquid may play a larger role in the potential health impacts.

A recent study by Liqiao et al. examining the emissions and dynamics of EC aerosols discovered that e-liquid composition has implications on $PM_{2.5}$[111]. $PM_{2.5}$ emission factor was negatively associated with PG and its fastest decay was observed when PG/GLY ratios were 100/0. In addition, nicotine (2.4%) reduced particle loss rate for $PM_{2.5}$ when a greater percentage of PG was available[111]. In these cases, PG and nicotine seem to impact $PM_{2.5}$ which could have an effect on deposition pattern after inhalation. Moreover, deposition of $PM_{2.5}$ on alveoli can trigger release of proinflammatory mediators, vasoactive molecules, and reactive oxygen species into the bloodstream inducing thrombogenesis[12]. In theory, high $PM_{2.5}$ levels in this study may suggest lower PG content and greater nicotine concentration which dependent on the temperature, oxidative conditions, and presence of metals implicate nicotine as an important player in ECs and warrants further investigation into the chemical characterization of EC aerosols. Overall, these findings do suggest the potential for ECs to induce adverse health effects, however further research is needed to ascertain the level in which $PM_{2.5}$ from ECs may induce CVD.

5.3 Changes in Heart Rate Variability

This study detected clear decreases in both SDNN and RMSSD indicative of reductions in HRV during EC exposures suggestive of a poor cardiac prognosis. While time domain parameters mainly reflect non-specific data, they do reflect the oscillatory activity generated by the different physiological control systems[95]. Both SS and PS activity contribute to SDNN during 24-hr recordings, however, in short-term recordings, the primary source of the variability is PS-mediated[95,112]. Hence, reductions observed in this study elude to shorter cycle lengths by which the amount of variation between normalized RR intervals are decreased. This is further bolstered by similar findings in RMSSD likewise modulated by parasympathetic response. Thus, reductions in this parameter indicate a decrease in PS activation suggestive of a change in autonomic variability.

Conversely, significant differences with pNN_6 values increasing during EC use appear to contradict what is observed in the other time domain parameters. Previous reports have indicated a strong correlation between RMSSD and pNN_6 parameters and are thought to represent a high degree of parasympathetic activity[114]. However, studies indicate that the RMSSD method is a better parameter for evaluating HRV than pNN_X because of its more robust statistical properties[95,112]. In addition, pNN_6 values were highly variable between trials and mice with no extreme outliers removed prior to statistical analysis. Even so, its significant increase in EC as compared to air and PBS ($p< 0.001$) cannot be easily dismissed. Therefore, further investigation into the reliability of this parameter is needed. Collectively, more reliable measures, SDNN and RMSSD, provide evidence for reductions in autonomic variability and may have implications in cardiovascular risk.

Frequency domain parameters were also analyzed in the context of HRV and taken into consideration to provide a clearer depiction of ANS modulations from EC exposure in C57BL/6 mice. This study demonstrated a significant increase in log (LF) during EC exposures compared to the combined air event suggesting sympathetic predominance. This finding is similar to those reported by Moheimani and colleagues[14]. However, when measuring log (HF), a direct physiological measure for parasympathetic activity[3,96], an increase was also observed, yet its magnitude was less than that of log (LF) (0.51 vs. 1.33). While LF power reflects physiological changes in sympathetic activity, it is also influenced by changes in vagal tone[90,112,113]. Therefore, the presence of some parasympathetic activation is not abnormal and has been reported in previous animal studies[113]. Indeed, this may be due to the fact that the degree of influence of the parasympathetic branch is thought to be greater for rodents than humans[113]. In this case, one possible reason may exist for the difference in results between mice in this study and human subjects in Moheimani et al.'s. In particular, rodents have reduced neuronal firing with inspiration and enhanced activation during expiration compared to larger mammals[113]. While respiratory rates were not measured in this study, these differences likely impact HF as it corresponds to HR variations related to the respiratory cycle.

In contrast, the significantly lower ratio of the spectral power in the LF and HF bands (LF/HF), a quantitative measure suggestive of sympathovagal balance suggests predominance of the PS. Yet, this value was not significant when comparing grouped EC vs combined air and its magnitude was relatively smaller. This could be due to a small sample size but previous researchers have challenged the belief that LF/HF measures truly signify the balance between the SS and PS[90,95,112]. During short-term recordings, respiration mechanics and resting HR (which can be affected by locomotor activity[114]) can create uncertainty regarding the contributions these

ANS branches may give to the LF/HF ratio[112]. In reality, LF power is not a pure index of SS drive as PS as previously mentioned, and blood pressure regulations via baroreceptors can contribute to the variability measures in this frequency band during resting conditions[86,112,113]. Collectively, more investigation in regard to locomotor activity, blood pressure, and respiratory rates will be required to truly ascertain sympathovagal balance and overall contributions of the SS and PS.

Decreases in HR during both EC exposures as compared to air and PBS suggest the prevalence of vagal activity, in contrast to previous reports[79-81]. Heart rate is largely under the control of the ANS[95]. Sympathetic influence on HR is mediated by epinephrine and norepinephrine release that leads to acceleration of electrical conductivity increasing AV conduction velocity and contraction of both atria and ventricles[115]. Parasympathetic activation mediates HR via acetylcholine and in general opposes effects produced by SS activity inhibiting AV node conduction[115]. Therefore, sympathetic activity would have a positive chronotropic effect not seen in this study. With respect to ECG parameters, QRS complex values indicate minor differences with no real changes between events suggestive of normal ventricular depolarization. While, shorter ST segments observed may indicate myocardial ischemia or infarction, statistical analysis will need to be utilized to determine whether differences between Air and EC exposures are not by chance. Furthermore, QT data may not be reliable as this parameter varies with HR and should be corrected for.

5.4 HRV and Experimental Design Considerations

We also considered how environmental changes could inadvertently affect heart rate with the mind to develop this model specifically for detecting changes in heart rate variability. The

average murine HR ranges between 500 –700 bpm depending on various environmental factors[116-118]. Baseline events indicate a 1-hour period where mice are initially introduced to exposures chambers and kept housed within to regulate HR. While not shown, temporal profiles of HR and temperature show uptake of these values during initial placement in the chamber, a normal behavioral issue. Overall, the mean HR during baseline was approximately 638 (2.08, SEM) for all mice and trials, falling within the normal range. This should have been comparable to values within the air event however, during the control exposure HR dropped to 578. Furthermore, almost all HRV parameters indicated a significant increase in Baseline in comparison to the control indicating high variability. This suggests time set for acclimation should be increased in future experiments to reduce heart rate levels comparable to the control.

Notably, physical characterization of PBS aerosols showed similar trends albeit at decreased magnitudes to EC aerosols. Even though PBS was initially included to correspond to previous animal models using this solution or saline , larger estimates in all HRV parameters in EC vs. PBS than EC vs. air were also reported (Table 2 and 3). Thus, PBS was compared to air to determine whether a significant difference existed between these controls. Excluding pNN6, all HRV parameters showed significantly higher magnitudes ($p < 0.001$) in favor of PBS. This could be due to its high salt concentration in comparison to air, and while useful for in-vitro work, that may not be the case for *in-vivo* aerosol generation systems. However, chemical characterization is needed as mentioned above.

Time series plots demonstrated EC aerosol particles remained within the chamber at very low levels but did not approach zero. While lower than the air event itself, grouped EC events were compared to grouped post-EC in consideration of whether residual EC aerosols led to larger differences during the second EC exposure. In SDNN, RMSSD, and LF/HF, EC was

significantly lower than post-EC, whereas pNN_6, LF and HF showed increases in EC. However, these values were relatively comparable to EC vs. Air in all HRV parameters indicating EC aerosols neither remain in the chamber after exposures have stopped nor induce biological effects.

While grouped exposures and custom contrasts provide conflicting HRV results, individual events demonstrate that changes to HRV may be reliant on the episodic nature of the exposures. In Figures 6 and 7, primary EC events were either smaller or comparable in magnitude to the second EC exposure in both time and frequency domain parameters. Moreover, EC 1 was not significant compared to air for SDNN ($p = 0.139$) and RMSSD ($p = 0.095$). This indicates the possibility of inducing greater autonomic dysfunction with additional intermittent EC episodes following a more chronic exposure reported by Moheimani and colleagues[14]. Further research will be needed to demonstrate a clear indication of reduction in HRV in the context of chronic exposure.

5.5 Limitations

Data retrieved from this study are primarily from mice whose cardiovascular profile is quite different than humans. As previously mentioned, modifications of HRV are highly correlated to the intrinsic rate of the heart. Given that rodent hearts are less stable than humans, data obtained was highly variable with differences between mice and trials requiring extensive statistical analysis. In addition, this study implemented a small sample size that may have contributed to the high intra-mouse and inter-mouse variability. Nonetheless, our robust statistical model considered these by introducing a random variable for each HRV parameter. Moreover, fluctuations in heart rate not only depend on environmental factors but also activity

levels[116-118]. Consideration of activity in future HRV studies on freely moving mice are recommended.

One of the bigger limitations to this research was the lack of nicotine measurements missing from this study. Extensive research into nicotine pharmacokinetic profiles from EC chronicallly induced cycles of nicotinic acetylcholine receptor activation, leading to addiction and cardiac effects[3,89,91]. Additionally, associations between nicotine and EC use have been determined to induce increased sympathetic activation in chronic vapors[89,119] implicating nicotine as a key player in influencing changes of HRV. As this study did not measure nicotine levels, there is no accurate report of whether nicotine is a major proponent in changes to HRV observed from acute EC use. Nonetheless, present studies have begun to look at this key factor.

Additionally, time may be a confounder in this study, HR and temperature gradually decrease over the course of the events (baseline to post-PBS), which seem to suggest that time could be an unintentional factor in our experimental design. This may have ramifications for HRV derived from RR intervals, however, the extent of its impact is unknown yet.

Lastly, although freely moving whole body exposure systems make the implementation of human topography easier, we do not know the exact levels of EC inhaled by the subjects (blood levels were not measured). EC aerosol deposited on fur or remaining in the chamber have the potential for dermal or oral exposure. Additionally, issues with the exposure chambers resulted in loss of EC aerosol during the first exposures. Initial use of exposure chambers revealed locations in which aerosols could easily escape. While, loss of EC aerosol was unsubstantial and repaired for the duration of the study, further modifications of additional exposure systems should be considered.

6. CONCLUSION

With the perception of EC's "safety profile", their use has drastically risen in the past decade. Despite the exponential increase in EC research, their safety assumption has not been fully studied in the context of cardiac autonomic dysregulation. Thus, we aimed to detect the impact of acute ECs on ANS dysfunction in C57BL/6 mice using an *in-vivo* exposure system that could simulate real-life conditions. First, analysis of particle mass and number concentrations were comparable to previous research and demonstrate a bimodal distribution. Temporal profiles further support this and indicate the presence of ultrafine and fine particles that may be generated from pyrolysis, metals and volatile compounds; all of which have major repercussions for human health. In addition, high levels of $PM_{2.5}$ suggest a potential for adverse health effects as e-liquid composition can impact $PM_{2.5}$ respiratory deposition when inhaled.

Secondly, detected changes to time domain parameters also demonstrated the ability to adapt this system for the detection of HRV with the addition of telemetry devices in mice. Reductions in SDNN and RMSSD indicate a decrease in autonomic variability and imply reductions of parasympathetic predominance during EC exposures. With regard to frequency domain parameters, increases in log(LF) indicate sympathetic activation. Log(HF) was also increased however, this value was smaller than LF. As the parasympathetic branch has more influence in rodents than humans, the presence of HF does not negate our initial findings. Moreover, the controversy behind LF/HF during short-term recordings question the reliability of this factor to determine how physiological factors can modulate ANS. Further investigation into the role activity, blood pressure, and respiratory rates play in the frequency domain is required to understand the physiological method behind ANS modulation during EC exposure.

Additionally, trends observed showing greater effects in all HRV parameters during a second round of EC aerosol suggest that a more chronic EC exposure may provide a clearer representation of autonomic dysregulation in mice as observed in human studies. In conclusion, this study shows acute EC exposures decrease autonomic variability in mice as measured by HRV and demonstrates that ECs may not be as safe as previously assumed with implications for CV health.

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