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Cardiac Effects of Daily Inhalation of E-cigarette Vapor

A thesis submitted in partial satisfaction of the requirements
for the degree of Master of Science

in

Biology

by

Ashley Wen Du

Committee in Charge:

Laura Crotty Alexander, Chair
Shannon Lauberth, Co-Chair
Ashley Juavinett

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The thesis of Ashley Wen Du is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-chair

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Chair

University of California San Diego
2019

DEDICATION

I dedicate this thesis to my loving mother, father, and sister
who I would not be the person I am today without. Thank you for your
unconditional support.

EPIGRAPH

“Tell me and I forget. Teach me and I remember. Involve me and I learn.”
-Benjamin Franklin

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LIST OF ABBREVIATIONS

VEH – Vehicle mice

EV – E-liquid exposed mice

PG – Propylene Glycol

VG – Vegetable Glycerin

BP – Blood Pressure

HR – Heart Rate

HRV – Heart Rate Variability

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ACKNOWLEDGMENTS

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ABSTRACT OF THE THESIS

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by
Ashley Wen Du

Master of Science in Biology
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Professor Laura Crotty Alexander, Chair
Professor Shannon Lauberth, Co-Chair

As e-cigarette use continues to rise in popularity, many have questioned how e-cigarette vapor inhalation actually affects mammalian systems. A previous paper from our lab showed how 6 months of exposure to daily inhalation of e-cigarette vapor (EV) led to fibrosis and

alterations in both heart rate (HR) and blood pressure (BP) in our female mouse model. We hypothesis that this effect will be replicated in a male model. This hypothesis is supported by data that males are more susceptible to cardiac risk. HR variability (HRV) has been highlighted as a sign of cardiac dysfunction, with increased risk of adverse cardiac outcomes. These experiments were designed to evaluate HR, HRV, BP, and gene expression of mice exposed to nicotine containing e-cigarette vapor daily for 6 months, 3 months, 1 month, and 1 week compared to controls. 10 week-old C57BL/6 mice were obtained from Jackson Labs. Mice were placed into the full body SciReq inExpose system where they were exposed for 60min daily to freshly made EV. The e-liquid used contained 70% propylene glycol, 30% vegetable glycerin and 6 mg/mL nicotine. At least once weekly during each exposure time point, mice were measured for their heart rate (HR), heart rate variability (HRV), and blood pressure (BP). The mouse's temperature was taken to ensure temperatures were between 35-37C during observation. Two cuffs were placed on the tail to measure both BP (Kent Scientific) over 5-10min. The EMKA tech ECGtunnel was used to measure HR and HRV in 15 minute intervals both pre-exposure and post-exposure. The results from our data suggest that chronic inhalation of nicotine containing e-cigarette vapor (EV) affects cardiac function and gene expression. The fibrosis we saw in our female mice is also present in our 6 month male mice, showing that it is not a gender specific effect. These results imply that e-cigarette users may be at risk for adverse cardiac physiological effects.

INTRODUCTION

Increase in public interest

Although E-cigarettes have been on the market in America since 2006, public interest in these electronic nicotine delivery devices has only truly spiked over the past 10 years (Pepper & Brewer, 2014) . E-cigarette advertisements have skyrocketed on popular social media sites such as Youtube, Instagram, and Twitter (Pepper & Brewer, 2014). Celebrities have also been using these devices on movies and television shows (Pepper & Brewer, 2014). In 2008-2010, search inquiries of E-cigarettes have far surpassed other nicotine delivery devices such as nicotine replacement therapy, despite being on the market for a much shorter period of time (Ayers, et al., 2011). Wider exposure and advertisement of e-cigarettes is likely linked to increased public interest and use of these devices.

Risk for adolescents

With flavorings that are created and marketed to smell like candy, it is no surprise that these devices have been increasingly popular in adolescents. The numbers of middle schools and high schoolers using e-cigarettes has increased as much as 40% in high school and 80% in middle school from 2017-2018 (Gentzke et al., 2018). This is concerning as not much research has been done on how using these devices from adolescence to adulthood may affect the body. Before e-cigarettes had gained popularity in the 2000's, the percentage of high schoolers and middle schoolers smoking traditional cigarettes had decreased significantly (Jenssen, 2019). Public perception of smoking in the younger generation had changed, with adolescents no longer viewing smoking as an attractive thing to do (Jenssen, 2019). Unfortunately, e-cigarettes seem to have reversed this progress as adolescents are shown to not view e-cigarettes or JUULs as

similar to smoking cigarettes (Jenssen, 2019). JUULs, the newest form of an e-cigarette, contain 59mg/mL pods of nicotine (as much as a pack of cigarettes), that young users seem to not know about the nicotine content (Jenssen, 2019). Additionally, there is data that suggests that adolescents who use e-cigarettes are more susceptible to advancing towards traditional cigarettes in the future (Etter, 2018). This effect is seen even after adjusting for susceptibility for smoking due to family history or environment (Etter, 2018).

E-cigarettes marketed as safer than cigarettes

E-cigarettes are commonly marketed as a safer version of cigarettes. The basis for this claim often sounds convincing as e-cigarettes lack the tar and combustion found in tradition cigarettes. However, studies have shown that even low tar and smokeless tobacco products still release toxic compounds found in cigarettes, and at similar levels (Auer et al., 2018), . Heat-not-burn electronic cigarettes were created to heat up tobacco with no ash and no smoke. When analyzed through gas chromatography, it was discovered that compounds found commonly in traditional cigarettes such as volatile organic compounds, hydrocarbons, and carbon monoxide, were still found in the e-cigarette (Auer et al., 2018). In terms of overall health benefits, when examining a population of e-cigarette users after 4 years, it shown that switching to e-cigarettes does not decrease the risk of being diagnosed with smoking related diseases (M.E. et al., 2019).

E-cigarettes and smoking cessation

It has been suggested that e-cigarettes may be an avenue to help cigarette users quit smoking, but there is varying data on whether or not that is true (M.E. et al., 2019). In a 4 year study, the dual e-cigarette and tobacco users and tobacco only users smoked the same number of cigarettes daily (M.E. et al., 2019). However, similar studies have also shown that dual users

were more likely to stop cigarette use if using e-cigarettes and that smokers stopping e-cigarette and cigarette use were more likely to relapse.

Components of e-cigarette liquid

To analyze possible health issues of e-cigarette use, it is important to understand what it is in the e-liquid juice itself. The composition of e-cigarette liquid (EV) is made of vegetable glycerin (VG), propylene glycol (PG), nicotine, and additive flavorings. While these ingredients are deemed safe for consumption, they are not necessarily known to be safe for inhalation (Jenssen & Boykan, 2019). Nicotine is known to be an addictive substance, but the other ingredients should not to be ignored and assumed to be nontoxic. PG is a respiratory irritant and VG is known to form acrolein, a reactive compound that is an irritant to the eyes and skin (Jenssen & Boykan, 2019). Increases in VG is associated with larger vape clouds, increasing the desire to smoke and to vape in adult smokers (Vena, Howe, Cao, & King, 2019). Additionally, some effects are shown to be seen in e-liquid with flavorings, but not e-liquid with just nicotine (Glynos et al., 2018). This suggests that flavorings are also toxic depending on the additive, and there are thousands of types of flavorings all with different ingredients (Jenssen & Boykan, 2019). Additives are noted to be the main source of carbonyl emissions from e-cigarettes, and make up the most common type of liquid used (Auer et al., 2017).

Importance of examining BP, HR, HRV

Cigarettes are known to have a systemic effect on the body. Besides being harmful to the lungs, effects have shown to travel to the heart and affect cardiology as well. Typical effects include increasing aortic stiffness, blood pressure changes, and heart rate changes (Papathanasiou et al., 2013). It has been shown recently that these effects are also seen in e-

cigarettes. In e-cigarettes, chronic inhalation is associated with a decrease in heart rate, increase in heart rate variability, and elevated blood pressure when systemic fibrosis is present in a female mouse model (Crotty Alexander et al., 2018). Aortic stiffness, a marker for premature or accelerated development of cardiovascular disease, has also been shown to increase in female e-cigarette exposed mice (Farsalinos & Gillman, 2018). Additionally, e-cigarettes have also been shown to be associated with increase in sympathetic regulation and oxidative stress, with decreased vagal tone (Moheimani et al., 2015). Heart rate variability is associated with cardiac autonomic dysfunction and frequency of coronary events (10, 11, 12). It is also a predictor of mortality in patients diagnosed with myocardial infarction (Kudaiberdieva et al., 2007). Cigarette smokers tend to have a decreased heart rate variability when looking at SDNN and RMSSD (Barutcu et al., 2005). Thus, it is an important marker to study when looking into cardiology.

E-cigarettes association with inflammation

Our lab has discovered that the chronic use of e-cigarettes led to deleterious effects in a mouse model (Crotty Alexander et al., 2018). Mice vaped for 3 or 6 months and experienced fibrosis and increased inflammation systemically (Crotty Alexander et al., 2018). This experiment was designed to take a closer look into the effects on the heart. The purpose of this experiment is to look into the pathway of inflammation and ultimately fibrosis in the heart while exposing mice chronically to e-cigarettes, at shorter time points of 1 week, 1 month, and 3 months. We hypothesize that chronic e-cigarette inhalation it will alter heart function and increase inflammation.

RESULTS

Gene expression in fibrotic cardiac tissue

At 6 months of exposure, fibrosis is seen in EV mice but not Air and VEH mice, showing that this effect is driven by nicotine. Evidence for fibrosis is shown in percentage of area with trichome staining (figure 1B). EV mice are shown to have significantly higher amounts of collagen staining, a fibrotic marker, in comparison to Air and VEH mice. When gene expression is analyzed in the apex of the heart, *Col1a1* and *TGFb* are shown to have higher mRNA fold change when comparing EV to VEH and air groups (figure 2D). Both genes are involved in collagen production and are profibrotic genes (Liu, 2011). The upregulation of these two genes illustrates their role as a fibrotic gene in our model. Although amount of *Col3a1* expression is not shown to be different among the three groups, other labs have shown that depending on the pathway of fibrosis and the organ it is in, *Col1a1* or *Col3a1* may not both be upregulated (Bishop, 2002). Other inflammatory markers such as *IL-6* and *IL-18* have similar levels of expression across all groups (figure 2C). Additionally, CTGF, another mediator of tissue remodeling and fibrosis, has similar levels of expression across all groups (figure 2C). The lack of expression of these common inflammatory markers and fibrotic markers suggest that they were perhaps expressed in earlier time points of the exposure and may have induced the fibrosis. We will investigate this in our 1 week, 1 month, and 3 month experiments.

Acute effects of e-cigarettes on cardiac measurements

Acute effects of e-liquid inhalation on mice is determined through post-exposure measurements. These measurements were taken immediately within 1 hour after exposure. In our 1 week mice, heart rate and heart rate variability do not show significant differences at the end of

the week (figure 3A, B). However, day 1 post heart rate variability trends lower for EV mice in comparison to VEH and Air mice (figure 3C, D).

At 1 month, post exposure systolic blood pressure is significantly higher in EV mice than air and VEH mice (figure 5). Other measurements of HR, HRV, and BP were not significant. SDNN and RMSSD measurements had larger variabilities in EV than air and VEH mice (figure 5). Nicotine in EV juice may be increasing or decreasing HRV differently in each mouse, while air and VEH mice are having similar responses to the treatment. This would account for the lower variability in air and VEH.

The 3 month exposure time point shows larger variability in EV post blood pressure measurements in both systolic and diastolic measurements (figure 6). In contrast, the air and VEH mice have tight BP values. Other post exposure measurements of HRV and HR do not show any trends of significant changes between groups.

Chronic effects of e-cigarettes on cardiac measurements

Chronic effects were determined with pre-exposure measurements. These measurements are taken approximately 24 hours after exposure, and right before the next exposure. The 3 month time point shows no significant changes in cardiac measurements. HRV measurements of RMSSD and SDNN are trending upwards in EV mice at the end of exposure (figure 6A). This is effect is not seen at earlier time points, and may even be the opposite of the 1 month measurements. At the 1 month time point, the HRV measurements are trending upwards from Air and VEH measurements (figure 3C, D). Additionally, both mouse body weight and heart to body weight ratios show no difference between all three groups at the end of 3 months.

Potential gene pathway towards fibrosis

Our experiments show significant changes can be detected in mice even with exposure time points much shorter than 6 months. Exposures as short as a 1 week time point are enough to drive gene expression changes. At one week of exposure, fold mRNA for transforming growth *TGFb* and *Col3a1* trend higher in EV mice when compared to Air and VEH mice. Both are proinflammatory and profibrotic genes affecting tissue regeneration and remodeling, and may be an early start to fibrosis. Additionally, *CTGF* fold mRNA is statistically higher in EV mice than both air and VEH mice. *CTGF* is another mediator of tissue remodeling and fibrosis and is downstream of *TGFb*. *TGFb* is not statistically higher in EV mice when compared to Air or VEH mice. This is suggestive that it was activated at an earlier time point and possibly downregulated after activation of *CTGF*.

At 1 month of exposure, gene changes continue to progress. *IL- 6* is significantly upregulated in EV mice compared to VEH and Air mice. This is both a proinflammatory and anti-inflammatory gene depending on the context of its activation. *CCL2* is statistically higher in EV mice when compared to both Air and VEH mice, while *CCL3* is trending up in EV mice but not significant. Nicotine is upregulating these proinflammatory genes in EV mice. *LIF*, a proinflammatory or anti-inflammatory gene, is statistically higher in EV.

CCN1 is significantly higher in VEH mice than Air and EV mice. This is surprising as with our 1 week mice, *CTGF* was significantly higher in VEH mice than Air and EV mice. More research needs to be looked into why this is the case. *CCN1*, also known as *Cyr61*, is significantly higher in VEH mice when compared to Air and EV. This is a marker of increased inflammation and decrease collagen. VEH mice may be experiencing increased inflammation but no fibrosis due to this gene.

Table 1. Composition of the e-liquid each experimental group was exposed to daily. Propylene glycol (PG) and vegetable glycerin (VG) are the two main components of e-liquid.

	E-Liquid Composition	Nicotine Level
Air	N/A	N/A
VEH	70:30 PG/VG	N/A
EV	70:30 PG/VG	6 mg/ul

Table 2. Exposure schedule for 1 month and 3 month exposures. Measurements include both BP and HR/HRV measurements. This schedule was designed to ensure mice were exposed at the same time everyday, and were in full body restraints at least twice a week.

	Monday	Tuesday	Wednesday	Thursday	Friday
8:00AM	Pre-Air BP	Pre-VEH BP	Pre-Air		Pre-VEH measurements
9:00AM	Air + VEH exposure ; Pre-EV BP	Air + VEH exposure	Air + VEH exposure	Air + VEH exposure	Air + VEH exposure, Pre-EV data
10:00AM	EV exposure	EV exposure	EV exposure ; Post-VEH measurements	EV exposure	EV exposure; Post-Air data
11:00AM				Post-EV measurements, Weight mice	

Table 3. Summary of all gene expression and cardiac measurement trends. Values deemed significant have a p value of <.05 by two way ANOVA.

	6 Months	1 Week	1 Month	3 Months
Trending	EV vs Air ↓ TNFa ↓ IL-18 ↑ IL-6 ↑ CCL3	EV vs Air ↑ TGFb ↑ Col3a1 Day 1 Post ↓ SDNN ↓ RMSSD	EV vs Air ↑ CCL3 Week 4 Post ↑ SDNN ↑ RMSSD	Week 12 Pre ↑ SDNN ↑ RMSSD Week 4 Post ↑ BP variability
Significant	EV vs Air ↑ Col1a1 ↑ TGFb	EV vs Air ↑ CTGF	EV vs Air ↑ CCL2 ↑ IL-6 ↑ LIF Week 4 Post ↑ SBP VEH vs Air/EV ↑ Cyr61/CCN1 ↑ CTGF	

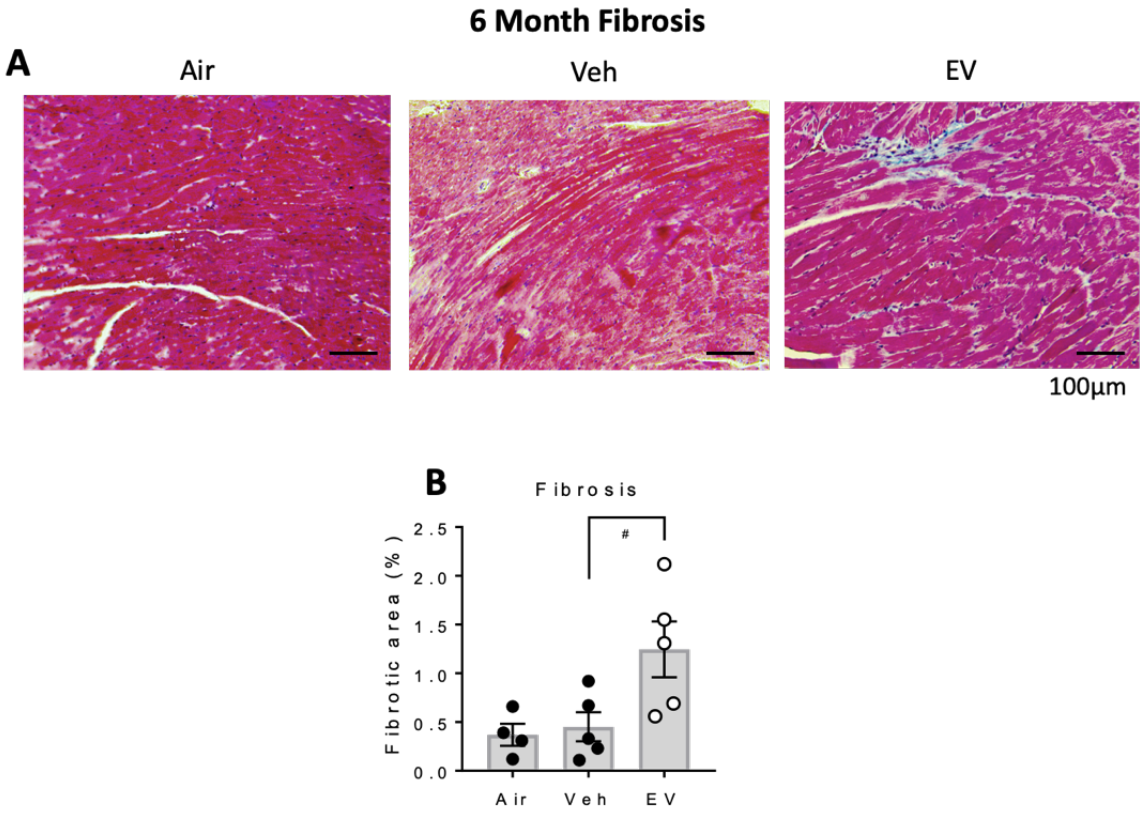

Figure 1. Mice that inhaled e-cigarette vapor with nicotine daily (EV) had significant changes in fibrosis of the heart apex. A) Heart apices were sliced and stained with trichrome staining. EV mice show significant collagen staining in comparison to Air and VEH mice. B) EV mice have a significantly higher fibrosis area than Air and VEH mice (n=6).

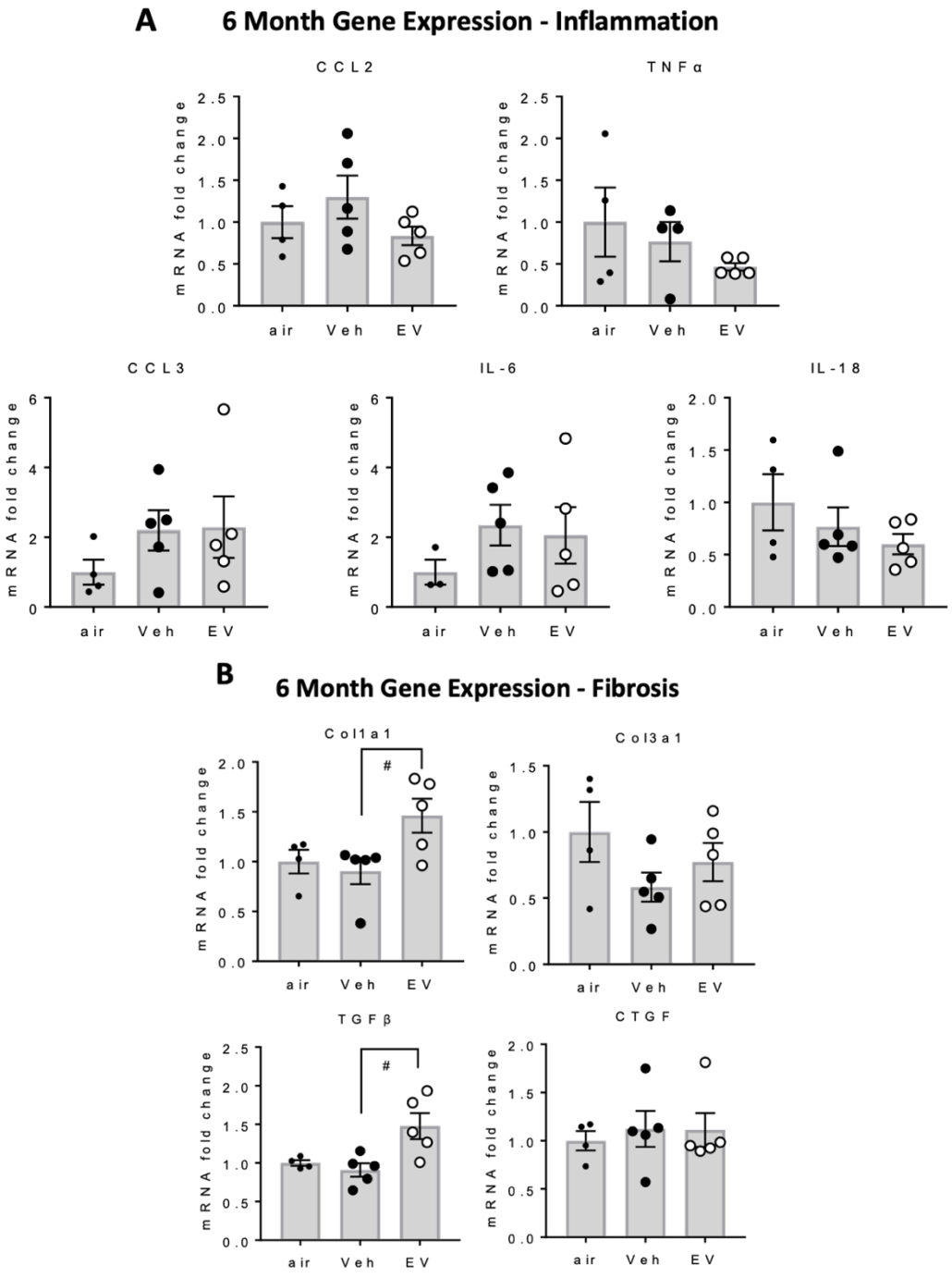

Figure 2. Mice that inhaled e-cigarette vapor with nicotine daily (EV) had significant changes in fibrotic marker gene expression but not inflammation gene expression. A) mRNA fold differences in common inflammation markers between each group of mice. B) mRNA fold differences in common fibrotic markers between each group of mice. Col1a1 and TGFb expression are significantly higher in EV mice than air and veh ($p < .05$).

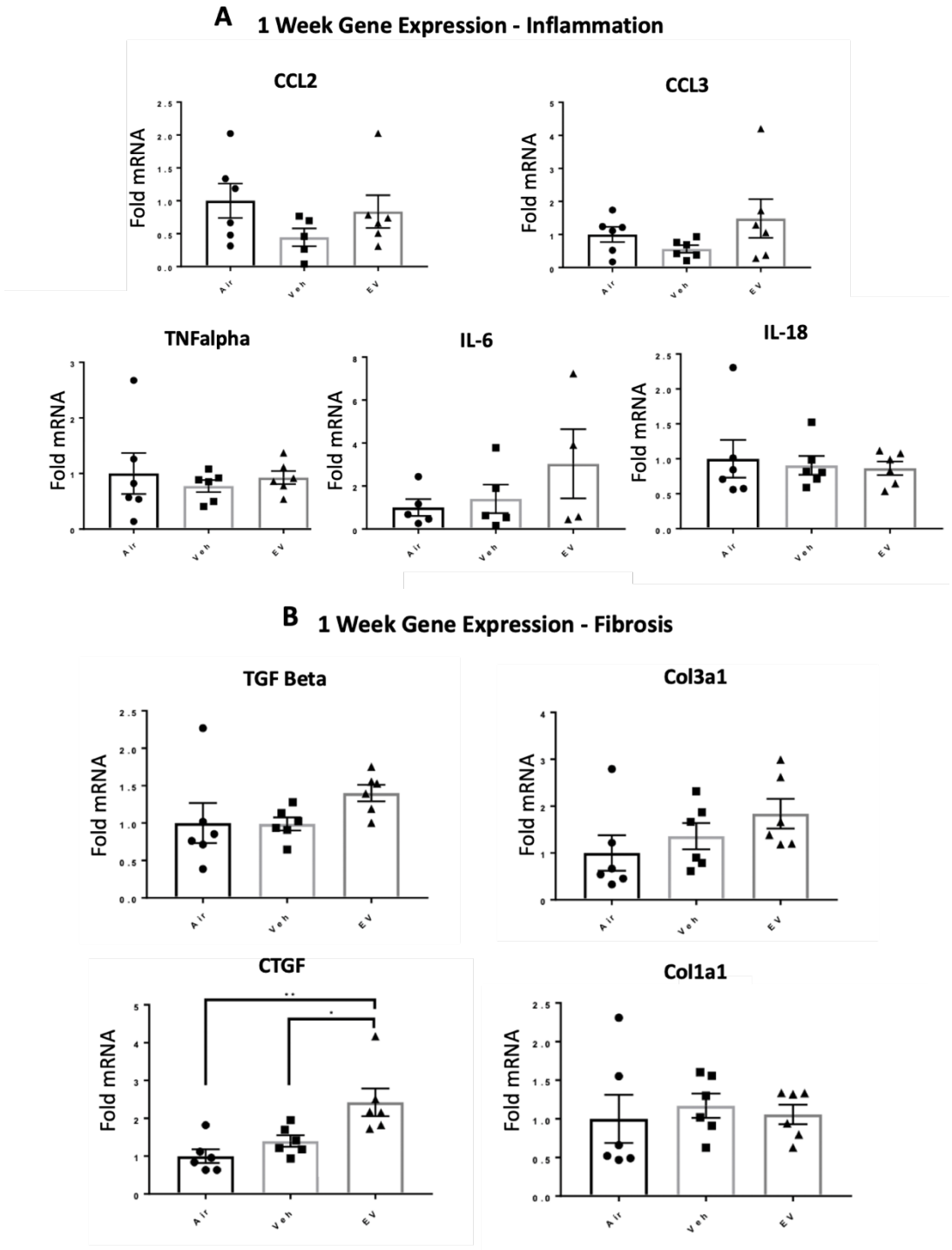

Figure 3. Mice that inhaled e-cigarette vapor with nicotine daily (EV) for 1 week (5 days consecutively) show a significant increase in CTGF. A) mRNA fold differences in common inflammation markers between each group of mice. B) mRNA fold differences in common fibrotic markers between each group of mice. CTGF expression is significantly higher in EV mice than air and veh ($p < .005$).

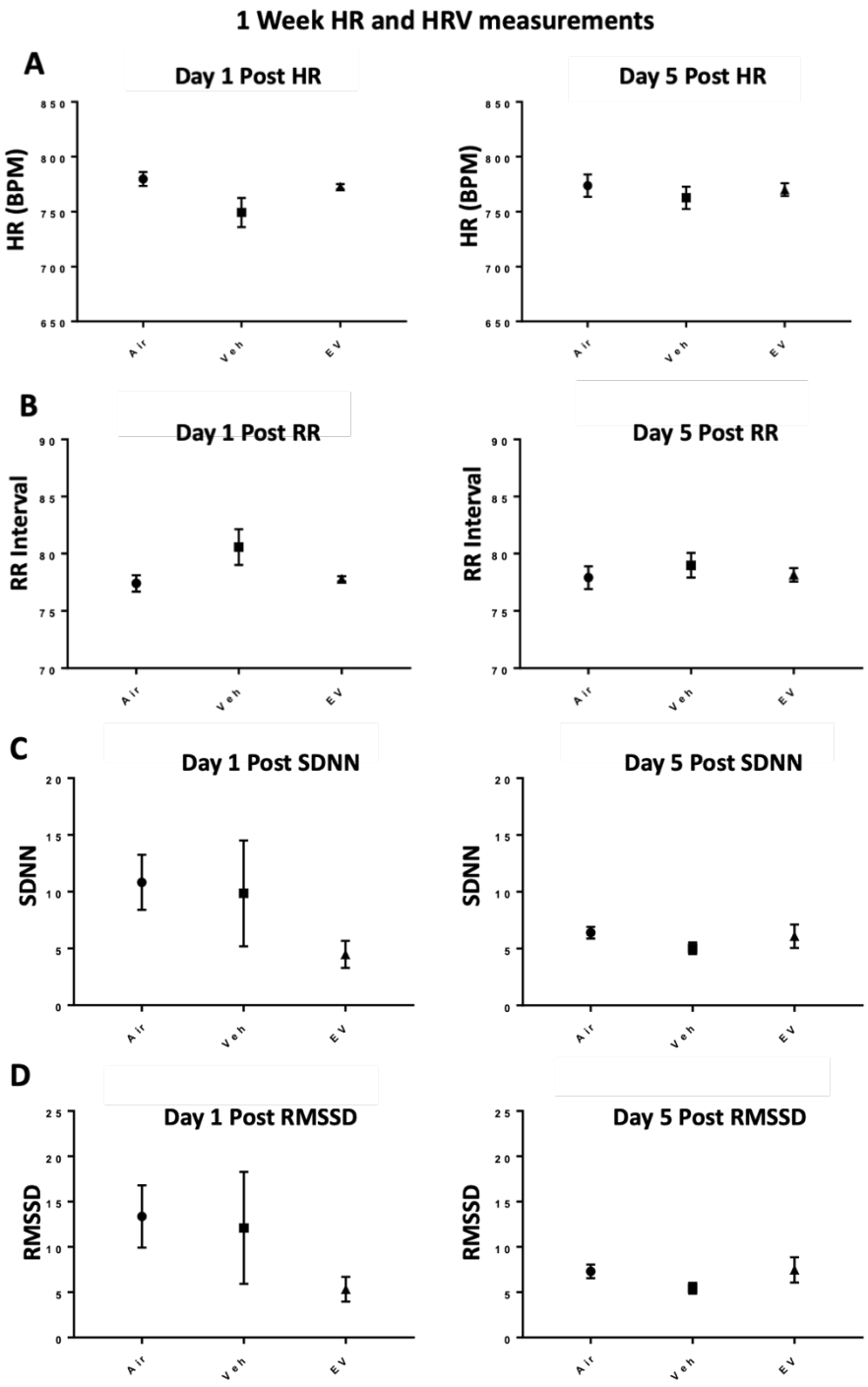

Figure 4. Mice that inhaled e-cigarette vapor with nicotine daily (EV) for 1 week (5 days consecutively) show no differences in HR or HRV. A) HR after the first exposure to e-liquid (day 1) and after the last exposure to e-liquid (day 5). B) RR after the first exposure to e-liquid (day 1) and after the last exposure to e-liquid (day 5). C) SDNN after the first exposure to e-liquid (day 1) and after the last exposure to e-liquid (day 5). RMSSD after the first exposure to e-liquid (day 1) and after the last exposure to e-liquid (day 5).

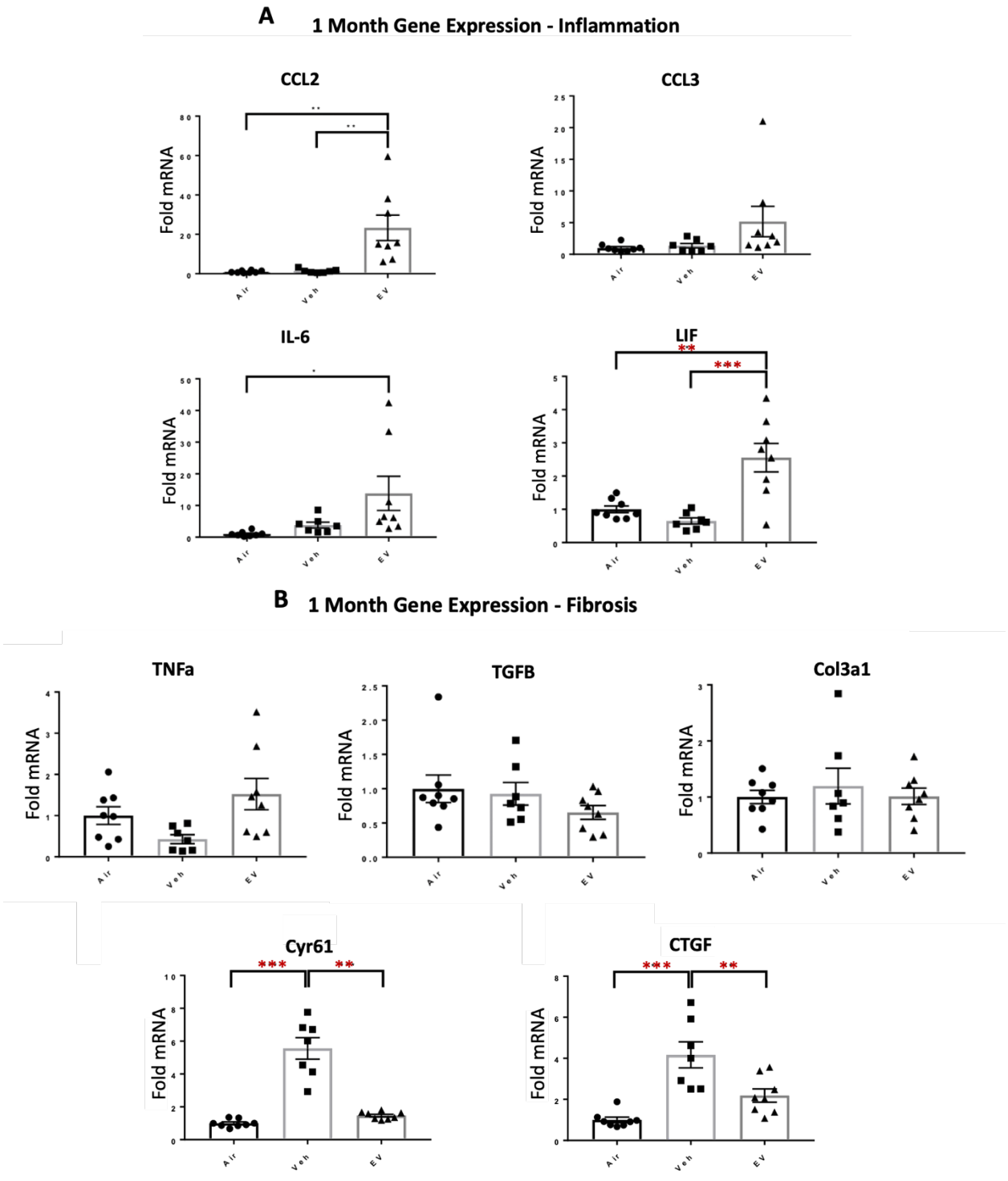

Figure 5. Mice that inhaled e-cigarette vapor with nicotine daily (EV) for 1 month show a significant increase in CTGF. A) mRNA fold differences in common inflammation markers between each group of mice. B) mRNA fold differences in common fibrotic markers between each group of mice. CTGF expression is significantly higher in EV mice than air and veh ($p < .005$).

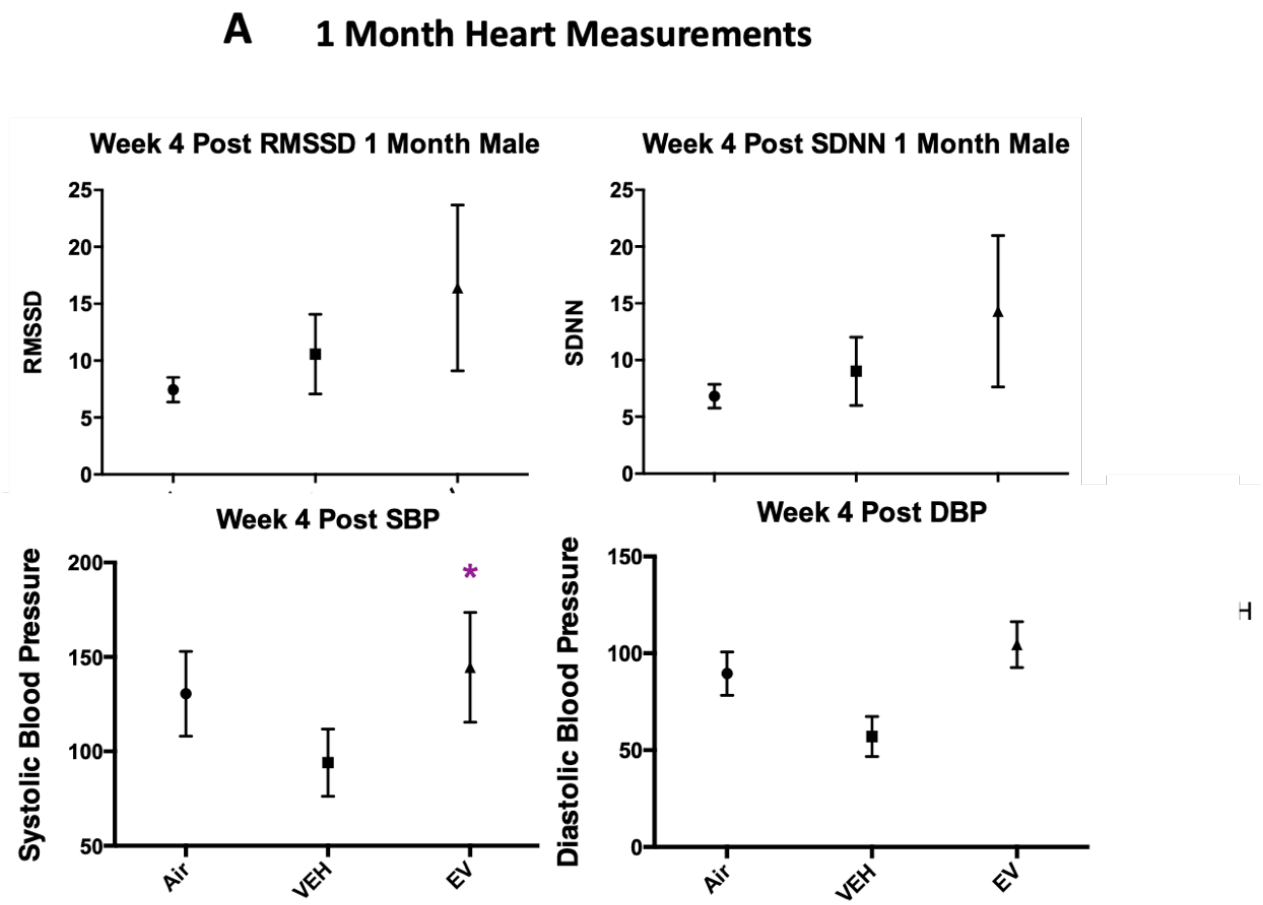

Figure 6. Mice that inhaled e-cigarette vapor with nicotine daily (EV) for 1 month did not have significant changes in heart HRV and BP in comparison to e-liquid without nicotine (VEH) or non-exposed mice (Air). A) Heart rate taken of VEH, Air, EV (n=6 each group) show no difference HRV measurements BP at week 12. B) BP of VEH, Air, EV do not show significant differences between groups.

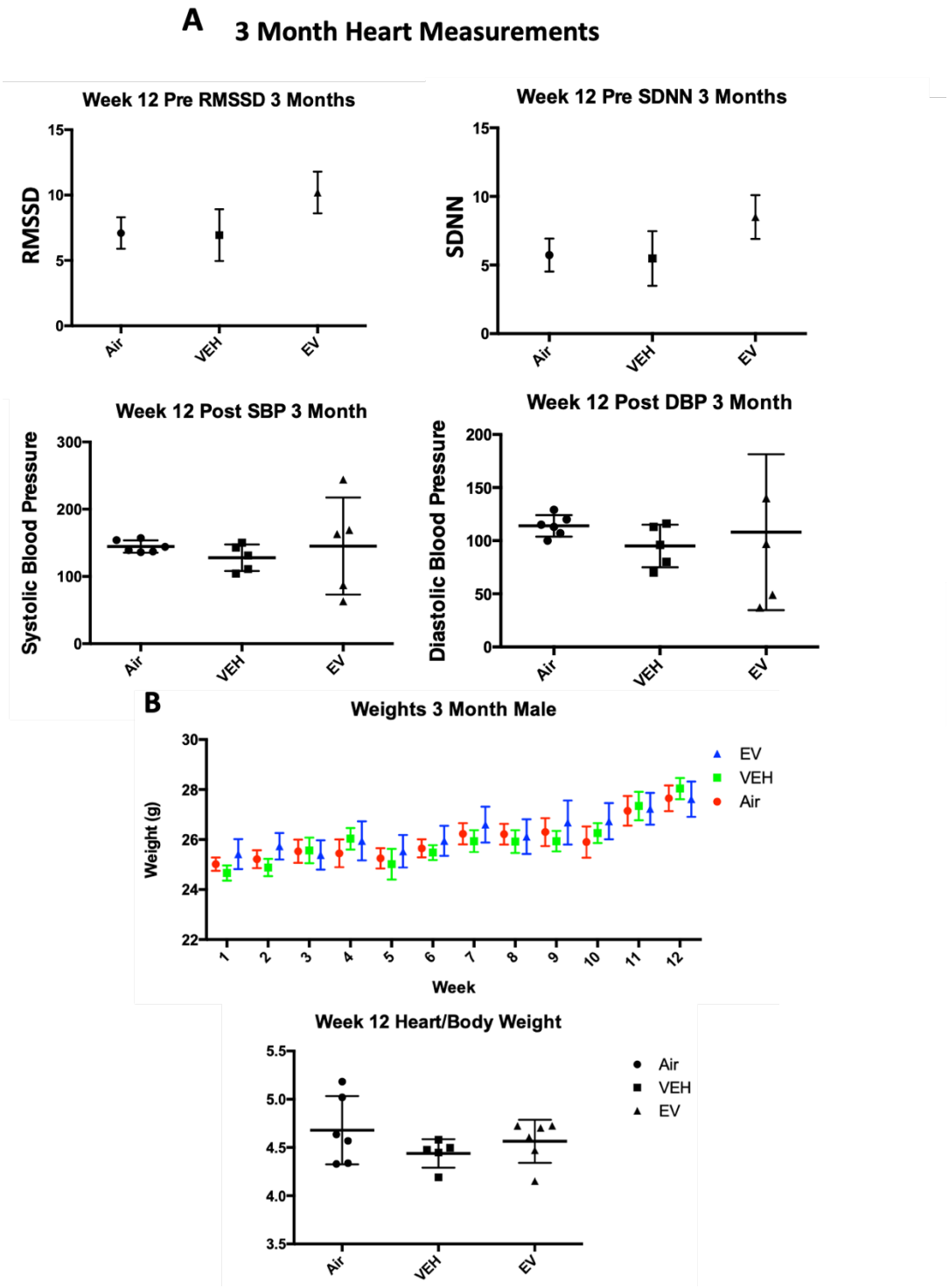

Figure 7. Mice that inhaled e-cigarette vapor with nicotine daily (EV) for 3 months did not have significant changes in heart measurements, weights, or heart to body weight ratio in comparison to e-liquid without nicotine (VEH) or non-exposed mice (Air). A) Heart rate taken of VEH, Air, EV (n=6 each group) show no difference HRV measurements BP at week 12. B) Weights of mice and heart to body weight ratio do not show significant differences between groups.

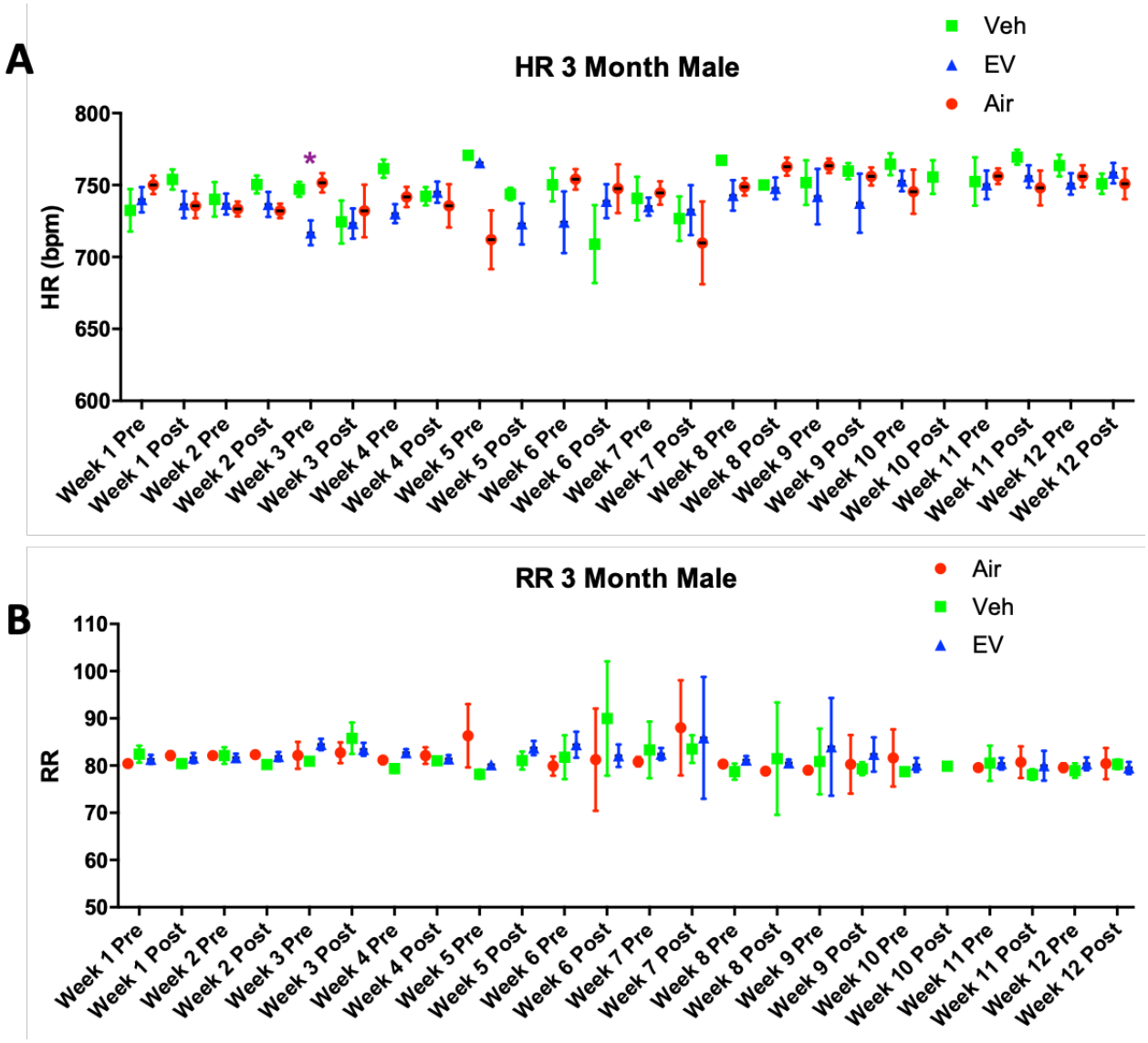

Figure 8. Mice that inhaled e-cigarette vapor with nicotine daily (EV) did not have significant changes in heart rate or RR interval distance in comparison to e-liquid without nicotine (VEH) or non-exposed mice (Air). A) Heart rate taken of VEH, Air, EV (n=6 each group) show no difference in both pre-exposure measurements and post-exposure measurements of heart rate, except at Week 3 pre measurements between VEH and EV (p value <.05). B) Heart rate taken of VEH, Air, EV (n=6 each group) show no difference in both pre-exposure measurements and post-exposure measurements of RR interval.

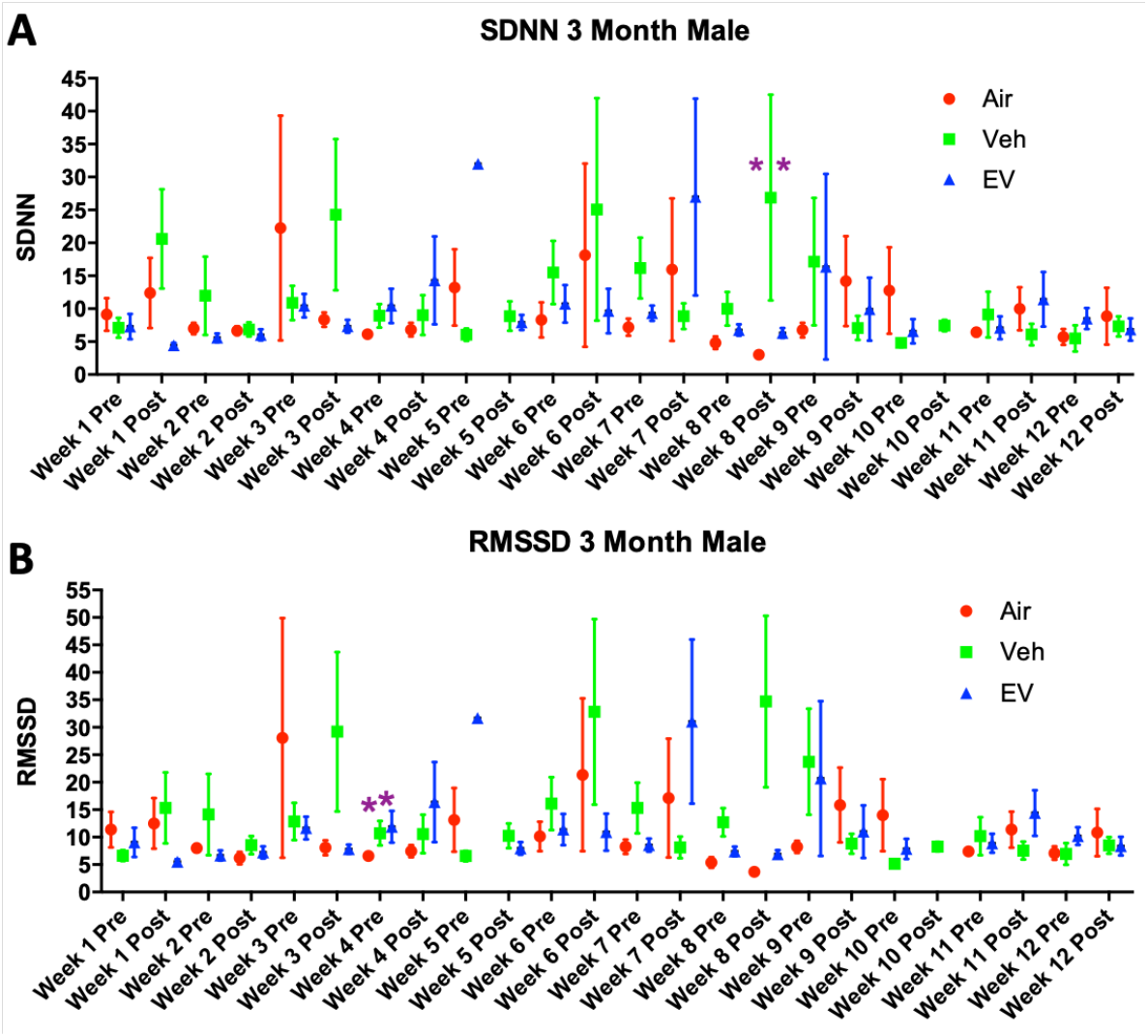

Figure 9. Mice that inhaled e-cigarette vapor with nicotine daily (EV) did not have significant changes in heart rate variability in comparison to e-liquid without nicotine (VEH) or non-exposed mice (Air). A) SDNN taken of VEH, Air, EV (n=6 each group) show no difference in both pre-exposure measurements and post-exposure measurements in variability, except at Week 8 post measurements between VEH and Air (p value <.05). B) RMSSD taken of VEH, Air, EV (n=6 each group) show no difference in both pre-exposure measurements and post-exposure measurements of variability.

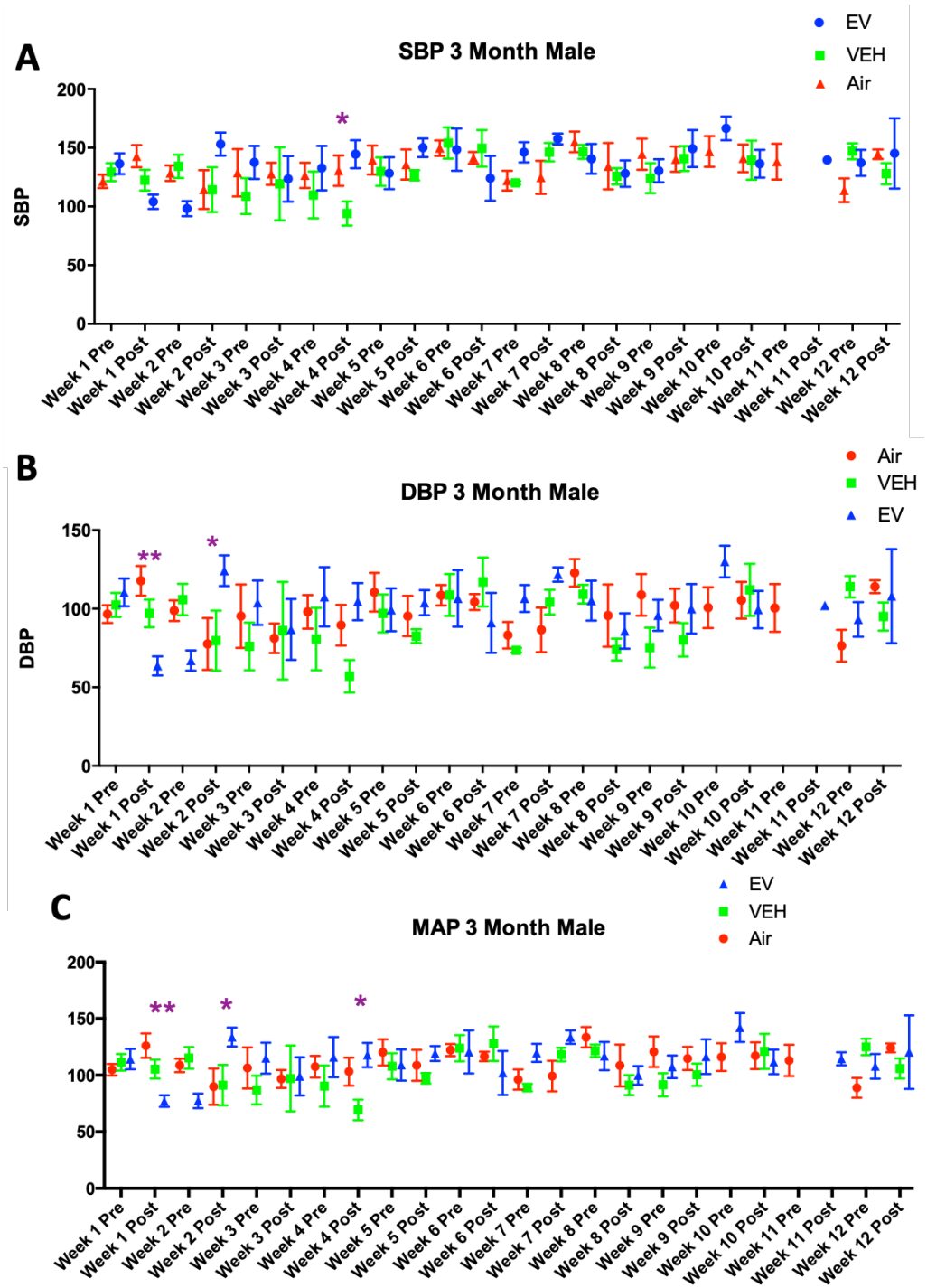

Figure 10. Mice that inhaled e-cigarette vapor with nicotine daily (EV) did not have significant changes in BP in comparison to e-liquid without nicotine (VEH) or non-exposed mice (Air). A) SBP taken of VEH, Air, EV (n=6 each group) show differences in Week 4 post between EV and VEH (p value <.05). B) DBP taken of VEH, Air, EV (n=6 each group) show differences in Week 1 post between Air and EV (p value <.005) and Week 2 post between EV and Air (p value <.05) C) MAP of three taken of VEH, Air, EV (n=6 each group) show differences in Week 1 post between Air and EV (p value <.05), Week 2 post between EV and Air (p value <.05), and Week 4 post between EV and VEH (p value <.05).

DISCUSSION

First, we wanted to examine if the fibrosis discovered in female mice previously in our lab was a gender specific effect. In humans and in mice, many diseases are gender specific or have gender specific pathways (Howell et al., 2017). Although we found fibrosis in both our female C57 mice and CD1 mice showing that it was not species specific, the work published was only done in female mice. Male humans and male mice in particular are shown to higher risk and incidence of cardiac disease (Udo & Grilo, 2015). Due to this, we hypothesized that fibrosis would be seen in our male mice as well. We thus subjected our male mice to the same conditions, 6 month exposure to e-cigarettes, as our female mice that we found fibrosis in. Looking into these male 6 month C57 exposed mice, we discovered fibrosis in EV mice exposed to nicotine and upregulation in fibrotic genes. The appearance of fibrosis in both models suggests that this pathways is not modulated by female hormones as it is discovered in both genders.

The 6 month male C57 showing expression of fibrotic genes but not inflammation genes, and having the phenotype of fibrosis suggests that we are already too late to catch this pathway to fibrosis. The fibrosis has already occurred and the fibrotic genes have already been expressed for a while. Our goal is to now look into shorter time points of 1 week, 1 month, and 3 month to observe the pathway of the inflammatory response that led to this fibrotic phenotype.

While e-cigarettes are commonly labeled as the healthier or safer alternative, our results show that they still have detrimental effects. Our mice are exposed for 1 week, 1 month, and 3 months from what is equivalent to an adolescent age through adulthood in our mice. Because e-cigarettes are so popular with adolescents, we determined this would be the most relevant age for human comparison. We use e-cigarettes that are commercially available and on the market for purchase to ensure they are translatable to human results.

A concerning result we discovered is e-cigarette inhalation affects gene expression even with only 1 week of exposure (5 days of exposure in a row). In our mice, inflammation markers are increased showing an early immune response to these toxins. Although HR, BP, and HRV do not show difference at this stage at the end of the weak, inflammation levels are high. The first day of exposure shows a change in EV mice with HR. Day 1 post heart rate variability trends lower for EV mice in comparison to VEH and Air mice (figure 3C, D). This is not surprising due to the nicotine in EV, and data showing acute EV exposure in humans affects heart rate (Weber, Sun, 2017).

While we did look at a variety of genes by qPCR, fibrotic pathways are known to be rather broad and can differ greatly depending on the organ system in question (Mukherjee & Sen, 1991). It is common for some pathways to include upregulation in some genes that are not seen in other organ systems (Eddy, 2014). By only checking 6 or so genes at every time point, there are many genes we still may have overlooked. As a next step, we plan to use RNAseq on our remaining mouse apices to give a much broader and more extensive overview. Our data does give insight into a potential pathway, but does not show enough. Further examination and studies is needed to determine this pathway.

Some critics about our setup include that these studies were done with a 1 hour a day exposure 5 days a week. While our e-cigarettes and system components are purchased from commercial sites available to humans, the exposure time is not necessarily accurate to how a normal human vapes. Humans tend to pick up a vape a few times an hour, usually for a few minutes (Villanti et al., 2017). This is very different from one entire hour a day. Additionally, our system is a full body chamber which allows the entire mouse's body to come into contact with the e-liquid vapor. Previous studies have showed that nicotine can be absorbed through the

skin (Maina et al., 2017). Thus, by allowing our mice to be exposed to nicotine through their skin, we may be subjecting them to a pathway that is not the same as nose only inhalation.

Although we expected to see HR, HRV, and BP changes at the 3 month time point, this was not the case. We had hypothesized that after acute inhalation, evaluated with post-exposure measurements, HR, HRV, BP may change in response to nicotine. Additionally, we believed that chronic inhalation, evaluated with pre-exposure measurements, would potentially show a different signal from acute measurements. This hypothesis was supported by human studies showing differences in blood pressure responses in chronic and acute inhalation after smoking a cigarette (Green 1986). However, we saw no clear signals throughout each weekly measurement leading up to 3 months and nothing significant at the end. In the future, we may extend our studies to 6 months, the time point at which we discovered fibrosis. Fibrosis is known to affect cardiology, and be affected by other factors such as hypertension (Weber 2017). It is likely that the cardiac fibrosis found in our female mice is what lead to the change in HR and BP that we discovered before. A longer time point with more chance of fibrosis occurring may lead to more visible changes in cardiac measurements.

In conclusion, our studies have shown that e-cigarettes do have a systemic affect like cigarettes do. Although they may appear to be safer, these effects should still be considered. With such an increase in interest from youths, potential hazards of long term use of these devices must be considered.

MATERIAL AND METHODS

Mouse exposure to E-cigarette Vapor

10 week-old male C57BL/6 mice were obtained from Jackson Labs. Mice were randomized into cages of three and three experimental groups: Air, VEH, and EV. Mice were placed into a full body chamber in the SciReq inExpose system where they were exposed for 60min daily 5 days a week to freshly made liquid for 1 week, 1 month, or 3 months. Liquid was refilled and pumps were cleared out every 15 minutes. The e-liquid used for VEH mice contained 70% propylene glycol and 30% vegetable glycerin. The e-liquid for EV mice contained 70% propylene glycol, 30% vegetable glycerin, and 6 mg/mL nicotine. Air mice were placed in the full body chamber with air pumping through it at the same rate and volume as VEH and EV exposures. The SciReq inExpose machine was calibrated daily for VEH and EV groups. The flow rate was set at 2 liters a minute, with 4 seconds of e-liquid every 20 seconds. Weights of each mouse were measured weekly. Cheek bleeds of mice were performed monthly to check cotinine levels in the blood.

Blood Pressure Measurements

Mice were acclimated to the ADInstruments CODA Non-Invasive Blood Pressure cuffs for three days consecutively, 15 minutes each day. Baseline measurements were taken after three days of acclimation and before the first e-cigarette exposure. Both pre-exposure and post-exposure measurements were taken at least once a week. The protocol used had 5 cycles of acclimation with 10 cycles per set, with 5 seconds between cycles. Tail cuff deflation time was set at 15s and the minimum volume was set at 15 microliters. All measurements were taken in body restraints and un-anesthetized. Mice were kept on a warming pad with the temperature set

to 38 degrees. Temperature of mice were taken before measurements through an infrared sensor at the base of the tail to ensure mice were kept at 32-35 degrees.

The ADInstruments CODA software was used to record and analyze the blood pressure measurements. Any measurements deemed unusable (fail tail volume, incorrect size, wrong shape, bad range) were not included in statistical analyses. Acclimation periods were not included in the analyses.

1 month and 3 month exposed mice were put in the ADInstruments CODA Non-Invasive Blood Pressure cuffs at least once a week for pre-exposure and post-exposure measurements. 1 week exposed mice were vaped in a staggered fashion of 15 minute intervals. Mice were placed 15 minutes in the ECGtunnel machine, exposed for 1 hour, then measured again with the ECGtunnel to ensure pre-exposure and post-exposure measurements.

Heart Rate Measurements

Mice were acclimated to the Emka ECGTunnel Chamber consecutively for three days, at 15 minutes each day. Baseline measurements were taken after three days of acclimation and before the first e-cigarette exposure. Both pre-exposure and post-exposure measurements were taken at least once a week for 15 minutes each. All measurements were taken in the tunnel restraint and while mice were un-anesthetized.

The ECGauto software was used to record and analyze the heart rate and heart rate variability measurements. Measurements were analyzed in 5 minute bins, using a sample ECG curve to detect acceptable HR beats. HRV was measured by RR intervals.

1 month and 3 month exposed mice were also put in the ECGtunnel at least once a week for pre-exposure and post-exposure measurements. 1 week exposed mice were vaped in a staggered fashion of 15 minute intervals. Mice were placed 15 minutes in the ECGtunnel

machine, exposed for 1 hour, then measured again with the ECGtunnel to ensure pre-exposure and post-exposure measurements.

Tissue Harvesting

Mice were exposed to Air, EV, or VEH immediately for an hour before harvest. One group was harvested at a time with mice staggered in start time to decrease the amount of time to get samples on ice. A terminal dose of Ketamine and Xylazine was injected as an anesthetic and heparin was injected afterwards. The abdomen was open for blood collection. Blood was collected intra-aortically and the trachea was cannulated. The BAL was collected and kept on ice. The heart was sterilely rinsed in PBS and sectioned into three sections: base, middle and apex. The middle and apex were snap frozen for protein analysis and the base was cryopreserved for sectioning. Lungs were cut into chunks and randomized into two tubes, one with RLT buffer, then snap frozen. The brain was removed whole and wrapped in foil to be put onto dry ice. The liver was separated into lobes, with one being snap frozen and one placed in fixative. One lobe of the kidney was snap frozen while another was placed in fixative. The tongue was cut off at the base and frozen. The head was placed in fixative. All snap frozen samples were stored in the -80C freezer. Fixed samples were kept in the cold room for 24 hours in fixative, then switched to 70% ethanol after 24 hours.

Statistical analyses

All data is presented with mean and standard error unless otherwise specified. Tests conducted were 2-way ANOVAs or t-tests using the Graph Pad Prism 6 software. ECGauto software was used to calculate HR and HRV data.

REFERENCES

Auer, R., Concha-Lozano, N., Jacot-Sadowski, I., Cornuz, J., & Berthet, A. (2017). *Heat-Not-Burn Tobacco CigarettesSmoke by Any Other Name*. *6*(p 2), 4–6.

Ayers, J. W., Ribisl, K. M., & Brownstein, J. S. (2011). Tracking the rise in popularity of electronic nicotine delivery systems (electronic cigarettes) using search query surveillance. *American Journal of Preventive Medicine*, *40*(4), 448–453. https://doi.org/10.1016/j.amepre.2010.12.007

Barutcu, I., Esen, A. M., Kaya, D., Turkmen, M., Karakaya, O., Melek, M., Esen, O., Basaran, Y. (2005). Cigarette smoking and heart rate variability: Dynamic influence of parasympathetic and sympathetic maneuvers. *Annals of Noninvasive Electrocardiology*, *10*(3), 324–329. https://doi.org/10.1111/j.1542-474X.2005.00636.x

Bishop, J. (2002). Regulation of cardiovascular collagen synthesis by mechanical load. *Cardiovascular Research*, *42*(1), 27–44. https://doi.org/10.1016/s0008-6363(99)00021-8

Crotty Alexander, L. E., Drummond, C. A., Hepokoski, M., Mathew, D., Moshensky, A., Willeford, A., Das, S., Singh, P., Yong, Z., Lee, J., Vega, K., Du, A., Shin, J, Javier, C., Tian, J., Brown, J., Breen, E. C. (2018). Chronic inhalation of e-cigarette vapor containing nicotine disrupts airway barrier function and induces systemic inflammation and multiorgan fibrosis in mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *314*(6), R834–R847. https://doi.org/10.1152/ajpregu.00270.2017

Eddy, A. A. (2014). Overview of the cellular and molecular basis of kidney fibrosis. *Kidney International Supplements*, *4*(1), 2–8. https://doi.org/10.1038/kisup.2014.2

Etter, J. F. (2018). Electronic cigarette: A longitudinal study of regular vapers. *Nicotine and Tobacco Research*, *20*(8), 912–922. https://doi.org/10.1093/ntr/ntx132

Farsalinos, K. E., & Gillman, G. (2018). Carbonyl emissions in e-cigarette aerosol: A systematic review and methodological considerations. *Frontiers in Physiology*, *8*(JAN), 1–14. https://doi.org/10.3389/fphys.2017.01119

Gentzke, A. S., Creamer, M., Cullen, K. A., Ambrose, B. K., Willis, ; Gordon, Jamal, A., & King, B. A. (2018). *Morbidity and Mortality Weekly Report Vital Signs: Tobacco Product Use Among Middle and High School Students-United States, 2011-2018*. *68*(6), 8. Retrieved from https://www.cdc.gov/tobacco/data_statistics/surveys/nyts/index.htm.

Glynos, C., Bibli, S.-I., Katsaounou, P., Pavlidou, A., Magkou, C., Karavana, V., Topouzis, S., Kalomenidis, I., Zakynthinos, S., Papapetropoulos, A. (2018). Comparison of the effects of e-cigarette vapor with cigarette smoke on lung function and inflammation in mice. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *315*(5), L662–L672. https://doi.org/10.1152/ajplung.00389.2017

Green, M. S., Jucha, E., & Luz, Y. (1986). Blood pressure in smokers and nonsmokers: Epidemiologic findings. *American Heart Journal*, *111*(5), 932–940. https://doi.org/10.1016/0002-8703(86)90645-9

Howell, M. D., Ottesen, E. W., Singh, N. N., Anderson, R. L., Seo, J., Sivanesan, S., Whitley, E., Singh, R. N. (2017). TIA1 is a gender-specific disease modifier of a mild mouse model of spinal muscular atrophy. *Scientific Reports*, *7*(1), 1–18. https://doi.org/10.1038/s41598-017-07468-2

Jenssen, B. P., & Boykan, R. (2019). Electronic Cigarettes and Youth in the United States: A Call to Action (at the Local, National and Global Levels). *Children*, *6*(2), 30. https://doi.org/10.3390/children6020030

Kudaiberdieva, G., Görenek, B., & Timuralp, B. (2007). Heart rate variability as a predictor of sudden cardiac death. *Anadolu Kardiyoloji Dergisi : AKD = the Anatolian Journal of Cardiology*, *7 Suppl 1*(31), 68–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17584685

Liu, Y. (2011). Cellular and molecular mechanisms of renal fibrosis. *Nature Reviews Nephrology*, *7*(12), 684–696. https://doi.org/10.1038/nrneph.2011.149

Flacco, M., Ferrante, M., Fiore, M., Marzuillo, C., La Vecchia, C., Gualano, M., Liguori, G., Fragassi, G., Carradori, T., Bravi, F., Siliquini, R., Ricciardi, W., Villari, P., Manzoli, L. (2019). Cohort study of electronic cigarette use: Safety and effectiveness after 4 years of follow-up. *European Review for Medical and Pharmacological Sciences*, *23*(1), 402–412. https://doi.org/http://dx.doi.org/10.26355/eurrev_201901_16789

Maina, G., Castagnoli, C., Ghione, G., Passini, V., Adami, G., Larese Filon, F., & Crosera, M. (2017). Skin contamination as pathway for nicotine intoxication in vapers. *Toxicology in Vitro*, *41*, 102–105. https://doi.org/10.1016/j.tiv.2017.02.022

Moheimani, R. S., Bhetraratana, M., Yin, F., Peters, K. M. P., Gornbein, J., Araujo, J. A., & Middlekauff, H. R. (2015). *Increased Cardiac Sympathetic Activity and Oxidative Stress in Habitual Electronic Cigarette Users: Implications for Cardiovascular Risk*. *20*(2), 163–178. https://doi.org/10.1007/s10741-014-9462-7.Natural

Mukherjee, D., & Sen, S. (1991). Alteration of collagen phenotypes in ischemic cardiomyopathy. *Journal of Clinical Investigation*, *88*(4), 1141–1146. https://doi.org/10.1172/JCI115414

Papathanasiou, G., Georgakopoulos, D., Papageorgiou, E., Zerva, E., Michalis, L., Kalfakakou, V., & Evangelou, A. (2013). Effects of smoking on heart rate at rest and during exercise, and on heart rate recovery, in young adults. *Hellenic Journal of Cardiology*, *54*(3), 168–177.

Pepper, J. K., & Brewer, N. T. (2014). Electronic nicotine delivery system (electronic cigarette) awareness, use, reactions and beliefs: A systematic review. *Tobacco Control*, *23*(5), 375–384. https://doi.org/10.1136/tobaccocontrol-2013-051122

Udo, T., & Grilo, C. M. (2015). *Cardiovascular Disease and Perceived Weight, Racial, and Gender Discrimination in U.S. Adults*. *40*(4), 1291–1296. https://doi.org/10.1097/CCM.0b013e31823da96d.Hydrogen

Vena, A., Howe, M., Cao, D., & King, A. (2019). The role of E-liquid vegetable glycerin and exhaled aerosol on cue reactivity to tank-based electronic nicotine delivery systems (ENDS). *Psychopharmacology*. https://doi.org/10.1007/s00213-019-05202-6

Villanti, A. C., Pearson, J. L., Glasser, A. M., Johnson, A. L., Collins, L. K., Niaura, R. S., & Abrams, D. B. (2017). Frequency of youth e-cigarette and tobacco use patterns in the United States: Measurement precision is critical to inform public health. *Nicotine and Tobacco Research*, *19*(11), 1345–1350. https://doi.org/10.1093/ntr/ntw388

Weber, K. T., Sun, Y., Gerling, I. C., & Guntaka, R. V. (2017). Regression of Established Cardiac Fibrosis in Hypertensive Heart Disease. *American Journal of Hypertension*, *30*(11), 1049–1052. https://doi.org/10.1093/ajh/hpx054