DEVELOPMENTAL OUTCOMES OF PRENATAL E-CIG AEROSOL VAPING

A Dissertation

by

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Electronic cigarettes (e-cigs) are tobacco products that have become popular among youth and young adults due to targeted advertising and misconceptions about their safety. The unfounded perception that e-cigs are less harmful than traditional cigarettes may result in the use of e-cigs during pregnancy. There are limited studies evaluating the effects of e-cig aerosol exposure on pregnancy in animal models and only a single report of gestational e-cig exposure in humans. To examine the impact of prenatal e-cig aerosol exposure on pregnancy and development we utilized a pregnant Sprague-Dawley rat model combined with a chronic, whole-body, environmental exposure to e-cig aerosols generated by commercially available e-cig atomizers. We found that exposure to e-cig aerosols containing nicotine significantly reduced fetal and neonatal growth, but aerosols without nicotine did not. Growth restriction was accompanied by reduced blood flow in the maternal uterine artery and fetal umbilical artery. Analysis of signature amino acid profile revealed altered concentrations in maternal and fetal plasma of animals exposed to e-cig aerosols with nicotine. Amino acid concentrations in the fetal lungs were altered by e-cig aerosols regardless of nicotine. RNA sequencing of fetal lung transcriptome showed altered expression after exposure to e-cig aerosols with and without nicotine. E-cig aerosols containing nicotine altered neonatal lung morphology and produced trends in respiratory mechanics that may increase the workload of breathing. Interestingly, e-cig aerosols with and without nicotine reduced the area of the pressure-volume loop during forced oscillation

techniques which may indicate increased atelectasis in neonatal lungs. The effects of prenatal e-cig aerosol exposure were more pronounced in animals exposed to aerosols containing nicotine, however, e-cig aerosols without nicotine were also found to effect the physiology of pregnancy. Further studies will be required to identify molecular mechanisms of e-cig aerosol induced alterations to pregnancy and development. The data presented in these studies lay a foundation for our understanding of prenatal exposure to e-cig aerosols by providing evidence that e-cig vaping during pregnancy can have deleterious outcomes for the developing offspring.

DEDICATION

I would like to dedicate my dissertation work to my family, and especially to my parents and grandparents for giving me the opportunity to pursue my degree. There are no achievements that would have been possible without your support. Your love and encouragement has allowed me to find passion in my work and for that I am forever grateful.

I would also like to dedicate this work to the memory of my grandfather, Raymond Orzabal. You taught me more than you will ever know and I am a better person because of you. Although you are not here to witness the completion of my doctoral degree, I know you would be proud of all that we have accomplished. You are with me always.

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Contributors

This work was supervised by a dissertation committee consisting of Professor Jayanth Ramadoss (chair) and Professor Stephen Safe of the Department of Veterinary Physiology and Pharmacology, Professor David Threadgill of the Department of Biochemistry and Biophysics, and Professor Rajesh Miranda of the Department of Neuroscience and Experimental Therapeutics.

Ultrasound equipment and training was provided by Dr. Threadgill. Processing of amino acid concentrations in Chapter 3 was conducted by Wenliang He of the Department of Animal Science. Dr. Guoyao Wu of the Department of Animal Science contributed to the interpretation of data in Chapter 3. Processing of RNA sequencing in Chapter 4 was conducted by Dr. Andrew Hillhouse of Texas A&M Institute of Genome Sciences and Society. Biostatistic analysis of RNA-seq data in Chapter 4 was conducted by Dr. Wesley Brashear of Texas A&M Institute of Genome Sciences and Society. Experimental execution and sample collection were assisted by Raine Lunde-Young, Vishal Naik, Dr. Jehoon Lee, Josue Ramirez, Dr. Katie Davis-Anderson, and Dr. Mathew Nemec of the Ramadoss Research Group.

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1. INTRODUCTION TO THE IMPACT OF ELECTRONIC CIGARETTE AEROSOLS ON PREGNANCY AND EARLY DEVELOPMENT*

1.1. Electronic Cigarettes

Tobacco product use during pregnancy is known to be a major factor contributing to numerous adverse outcomes that can affect both the mother and fetus (*1*). Electronic cigarettes (e-cigs) are one of the latest forms of tobacco products and have gained increasing popularity since their introduction in the United States in 2007. E-cigs are advertised as a harm reduction tool to help in the cessation of cigarette smoking (*2*). E-cigs can be highly addictive and have the capacity to deliver as much or more nicotine as traditional cigarettes (*3, 4*). The e-cig device is composed of a battery, heating element, and an e-cig liquid cartridge. E-cig liquid typically contains nicotine, flavorings, and humectants such as propylene glycol and glycerol which are vaporized by the heating element and inhaled by the user.

The perception that e-cigs are a safer alternative to traditional cigarettes because of reduced exposure to hazardous chemicals and tar may lead some cigarette smokers to switch to e-cigs during pregnancy. However, the effects of e-cigs on early human development are not currently understood and there is little evidence to support the claim

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that e-cigs are safer than traditional cigarettes. Vaping e-cigs changes the chemical makeup of the e-cig liquid, producing aerosols that contain a number of harmful byproducts including formaldehyde, trace metals, and small particulate matter (*5, 6*). E-cigs may also pose a threat to non-users through second-hand exposure to e-cig aerosols or by third-hand exposure through the accumulation of aerosol residues on objects in the vicinity of vaping. As the use of e-cigs continues to increase, so does the need for studies investigating the health effects of e-cigs on pregnancy and early development. Herein, we will review the most recent findings related to e-cig use and its effects on multiple facets of pregnancy and development.

1.2. Incidence of E-cig Use

According to the 2017 National Health Interview Survey (NHIS), approximately 47.4 million U.S. adults (19.3%) are currently using tobacco products of any kind, and an estimated 30% of these tobacco consumers are using both e-cigs and traditional cigarettes (*7*). While traditional cigarette smoking has diminished among adults in the U.S., there is still a need to reduce overall tobacco product consumption and exposure. Despite efforts to reduce the incidence of tobacco product consumption during pregnancy, global reports show that as much as 52.9% of daily smokers continue to use tobacco products while pregnant (*8*). Smoking during pregnancy exposes the fetuses to nicotine and other harmful chemicals *in utero* and raises the risk for adverse outcomes for the mother and child.

Since their introduction to the market, e-cigs are being used by both smokers and non-smokers, including teens and young adults. The 2018 National Youth Tobacco

Survey reports e-cigs as the most common form of tobacco product used among middle and high schoolers with 20.8% of high schoolers currently using e-cigs in 2018 (*9*). The popularity of e-cigs among younger generations and people of child-bearing age raises concern for their impact on pregnancy and fetal development. The addictive properties of nicotine and the perception that e-cig vaping is a safer alternative to traditional smoking may lead some individuals to use e-cigs during pregnancy. In a study conducted in 2015, 11.9% of pregnant women were categorized as current e-cig users in a cohort of 382 which may indicate a substantial risk for public health (*10*). There is limited data investigating the effects of e-cigs in pregnancy and with an increasing trend of e-cig use among vulnerable populations, it is necessary that short- and long-term consequences of e-cig vaping be identified.

1.3. Current Models

Due to the nascent stage of e-cig consumerism there are few studies that report the short-term health effects of e-cig vaping, and there are no examinations on the effects of e-cig use during pregnancy or long-term health effects of e-cigs in humans. For this reason, animal models are necessary for identifying potential health risks associated with e-cig use and pregnancy. To date, the small number of animal studies that have investigated the use of e-cigs during pregnancy report complications to fetal development ranging from physical morphology to major organ system development and function, having the potential to further impact adult health (Figure 1-1), and are summarized in three sections below. Recent animal models utilize several species including rodents, amphibians, and fish. A number of studies have also examined the

effects of e-cig liquid or condensed e-cig vapors on cell lines from different organs (*11-13*). Many of these studies propose that nicotine is the major component attributing to negative health outcomes for the developing fetus. Although studies have reported effects of nicotine replacement therapies such as dermal patches, gum, and inhalers in pregnancy, the current review is focused on e-cig exposure (*14-16*). Nicotine can easily pass into fetal circulation and can reach blood nicotine concentrations that are equivalent to maternal levels (*17*). Evidence that constituents other than nicotine in e-cig aerosols has also been reported to have a harmful effect on the developing fetus in animal models (*18-24*). The current literature on e-cig use in pregnancy demonstrates that e-cigs can potentially harm the fetus and necessitates tighter regulation of e-cig products and their use during pregnancy.

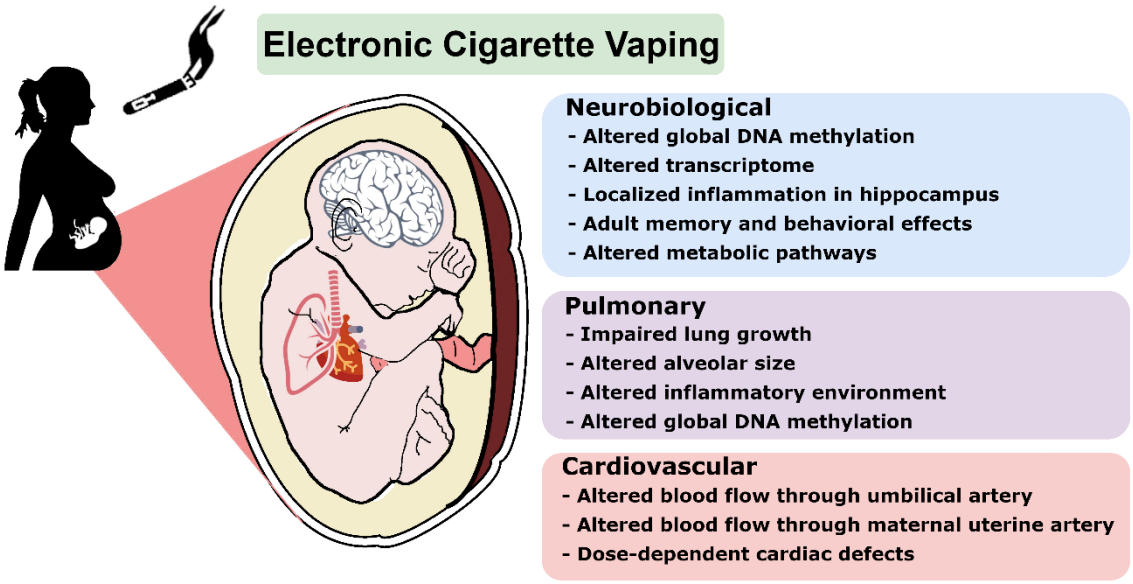

Figure 1-1 Effects of e-cig aerosols on Pregnancy and Early Development. Summary of potential health risks associated with e-cig use during pregnancy based on animal studies that have documented the effects of e-cigs on fetal development ranging from physical morphology to major organ system development and function. Reprinted with permission from (*25*).

1.4. Impact of E-cigs on the Cardiovascular System

During pregnancy, the workload on the maternal cardiovascular system increases dramatically. To compensate, significant vascular adaptations occur, especially in the uterine artery, the main vessel that delivers oxygen and nutrients to the fetoplacental compartment (*26*). Chronic nicotine exposure during pregnancy can impair maternal vascular adaptations and decrease uterine artery blood flow by approximately 40% in gestational animal models, effectively reducing oxygen and nutrient delivery (*27-29*). The effects of nicotine may permanently alter the intrauterine environment which could further compromise the physiological development of major fetal organ systems (*30*).

Animal models of nicotine exposure during gestation reveal that nicotine can have immediate and lasting effects on fetal and adult offspring cardiovascular health. In sheep, prenatal nicotine (25-30 μg/kg), delivered intravenously in late gestation resulted in increased arterial pressure and umbilical vascular resistance with diminished umbilical blood flow; and decreased heart rate accompanied by various arrhythmias (*28, 31*). In adult rat offspring, early life exposure to nicotine (6 mg/kg/day; osmotic minipump) resulted in vascular oxidative damage, increased contractility of the aorta, and reduced maximal dilation of the aorta in the male offspring (*32, 33*). These changes to the adult cardiovascular system further increase the risk for major cardiovascular diseases such as hypertension, atherosclerosis, and coronary artery disease later in life.

Although there are a number of studies investigating the effects of nicotine on the developing cardiovascular system, there are only two reports on the effects of e-cig aerosols. A recent study observed the effects of chronic e-cig aerosol exposure during and shortly after pregnancy (gestational day 5 to postnatal day 10) on maternal and fetal hemodynamics in a rat model (*34*). A custom-engineered vaping system was utilized to generate and deliver e-cig aerosols that mimic the aerosols produced by commercial e-cigs to dams and neonates (3 hr/day, 5 days/week). In animals exposed to e-cig aerosols containing nicotine, blood flow was significantly decreased in the maternal uterine artery (↓49.50%) and the fetal umbilical artery (↓65.33%) when compared to pair-fed control animals near the end of gestation. Decreased blood flow through these vessels was accompanied by a reduction in fetal and pup weight and crown rump length (Figure 1-2). E-cig aerosols without nicotine did not appear to have any significant effect on

hemodynamic measurements or growth parameters, however, other studies have shown that postnatal exposure to flavored e-cig aerosols without nicotine can significantly decrease offspring weight (*18*). In another experiment utilizing a zebrafish model, Palpant et al. demonstrated that e-cig aerosol extracts (16 mg nicotine/cartridge) can negatively affect overall heart development and function in the embryo, but to a lesser extent than traditional cigarette extracts (*35*). The cardiovascular effects of e-cig aerosols during pregnancy closely resemble those of tobacco smoke exposure during pregnancy and prenatal nicotine exposure, suggesting that much of the cardiovascular complications due to tobacco product use during pregnancy may be derived from nicotine.

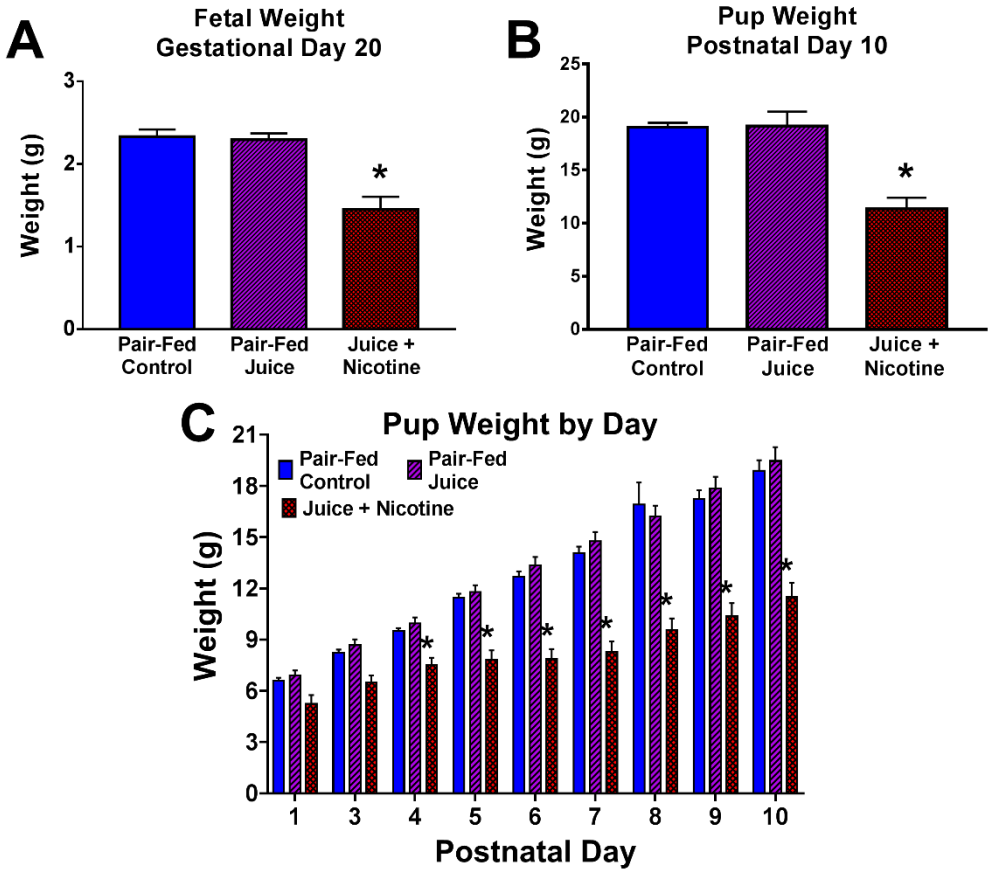

Figure 1-2 Effects of e-cig aerosols on fetal and postnatal growth. Following gestational and postnatal vaping, fetal and pup weight were measured on gestational day (GD) 20 and postnatal day (PND) 10, respectively, one day after the last vaping episode. (A) Mean fetal weight in the Juice + Nicotine group was decreased compared with Pair-Fed Control and Pair-Fed Juice groups ($P < 0.0001$). (B) Pup weight in the Juice + Nicotine group was decreased compared with both Pair-Fed Control ($P = 0.0002$) and Pair-Fed Juice groups ($P < 0.0001$). (C) Postnatal pup weight in the Juice + Nicotine group was decreased compared with the Pair-Fed Control and the Pair-Fed Juice Groups on PND 4-10. Values are mean + SEM, * indicates statistical significance, $P < 0.05$. Adapted with permission from (*34*).

1.5. Impact of E-cigs on the Pulmonary System

Prenatal nicotine exposure can alter fetal lung development and is implicated as a source of pulmonary dysfunction in offspring of women who used tobacco products

during pregnancy (*36*). In mouse (2 mg/kg/day; osmotic minipump) and rhesus monkey (1.5 mg/kg/day; osmotic minipump) models of prenatal nicotine exposure, offspring lung development and function reflected phenotypes that were similar to those found in human studies of children who were exposed to tobacco products *in utero* (*37-39*). Nicotine-induced disruptions to fetal lung development are thought to be mediated by an increase in nicotinic acetylcholine receptors (nAChRs) located on macrophages, epithelial lining cells, and fibroblasts by promoting an abnormal pattern of growth throughout the lungs (*38, 40*). In a mouse model, it was demonstrated that the fetal lung was most sensitive to nicotine-induced (2 mg/kg/day; osmotic minipump) changes during the period of gestation day 14 to postnatal day 7, similar to the later stages of lung development in humans (*38, 41, 42*). Histological examination of rhesus monkey fetal lung after prenatal nicotine exposure (1 mg/kg/day; osmotic minipump) showed decreased lung size and volume, with an increase in collagen, wall thickness, and alveolar volume (*43*). Observations on the effects of tobacco products in humans reveal that the reduced size and surface area of the lungs after gestational tobacco use restricted normal lung function by reducing respiratory compliance, forced expiratory flow, and the tidal breathing ratio (*1, 44*). Impaired lung function after nicotine or cigarette smoke exposure *in utero* is strongly correlated with incidence of sudden infant death syndrome (SIDS) (*45*). In addition to the consequences directly to the offspring of mothers who used tobacco products during pregnancy, it is now reported that there may also be consequences to the second generation of offspring due to epigenetic factors induced by nicotine exposure (*46*). The effects of gestational nicotine exposure on lung development

may be life-long and increase the risk for future respiratory complications, as well as impacting second generation offspring by epigenetic mechanisms.

The dangers associated with gestational tobacco product use and lung development are well documented and raise concern for e-cig use during pregnancy, however, there are only two studies that have investigated the relationship between early lung development and exposure to e-cig aerosols. To examine the effects of e-cig aerosols with or without nicotine on postnatal lung development in mice, McGrath-Morrow et al. utilized a whole body environmental exposure technique to expose neonatal mice to e-cig aerosols (18 mg/ml) from postnatal day 1 to 9 (*18*). In this study, they found that neonates exposed to flavored e-cig aerosols with and without nicotine decreased overall weight, suggesting that constituents other than nicotine in the aerosol may have harmful effects on offspring health. In the neonates that were exposed to e-cig aerosols with nicotine, postnatal alveolar growth was significantly impaired compared to a room-air control group as determined by mean linear intercept measurements taken from histological lung samples to measure the distance between gas exchange surfaces. Exposure to e-cig aerosols containing nicotine also decreased cell proliferation in the airspaces, but there was no evidence of apoptosis or oxidative stress among the three groups (Figure 1-3). In a more recent study conducted by Chen et al., gestational exposure to e-cig aerosols with nicotine (18 mg/ml; inhalation) and without nicotine altered the levels of pro-inflammatory cytokines within the lungs of adult offspring (*20*). This study also proposed that DNA methylation may be a potential mechanism for adverse effects on offspring health due to an increase in global DNA methylation in the

lungs of offspring that were exposed to e-cig aerosols with and without nicotine. It is important to note that the observations made in this study show that the effects were only partially due to nicotine, and that e-cig aerosols other than nicotine may still be harmful to the developing fetus. These studies support the notion that exposure to e-cig aerosols during early life can significantly disrupt lung development and growth which can increase the risk for later respiratory morbidities.

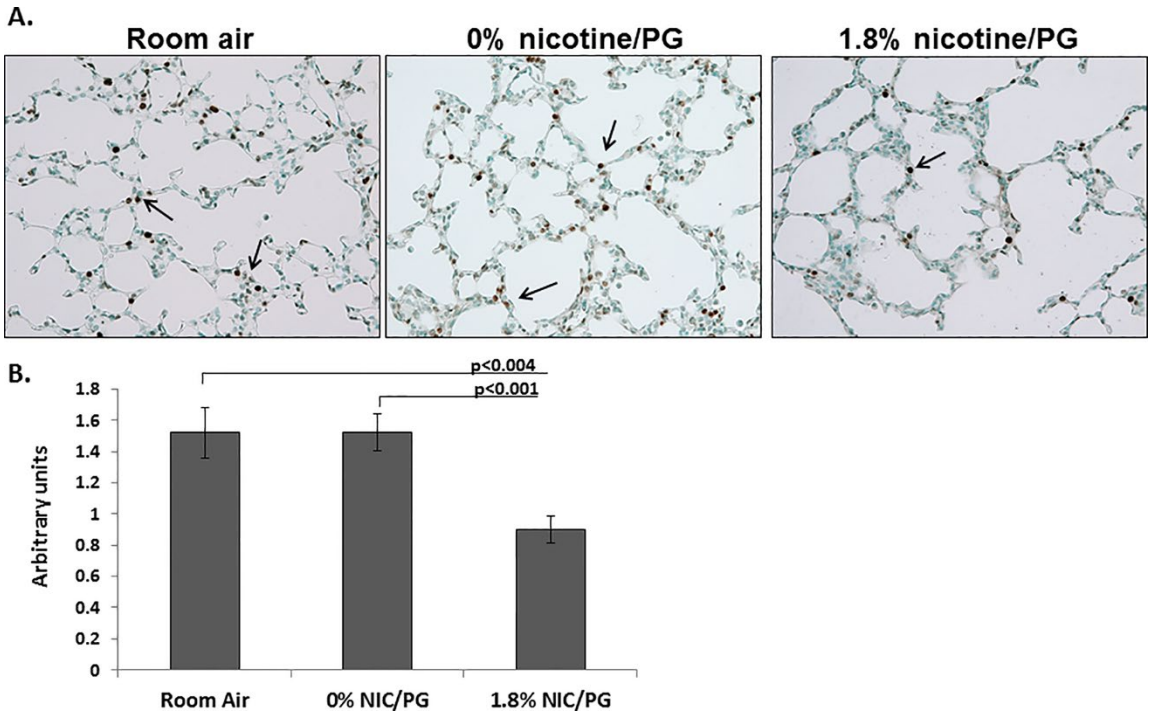

Figure 1-3 Decreased cell proliferation in airspaces of neonatal mice exposed to 1.8% nicotine/PG. (A) Arrows point to KI67 staining in the airspaces of 10 day old neonatal mice. (B) Quantification of KI67 staining showed significantly less KI67 staining in 10 day old neonatal mice chronically exposed to 1.8% nicotine/PG compared to room air and 0% nicotine/PG treated mice. (n = 8 per group; error bars reflect standard error of the mean; PG = propylene glycol). Adapted with permission from (*18*).

1.6. Neurobehavioral Effects of E-cigs

Since the effects of e-cig aerosols during pregnancy and early life are still largely unknown researchers are exploring many different components of development in order to understand the impacts of these next-generation tobacco products. The developing central nervous system is particularly sensitive to nicotine exposure, thus several studies regarding the effects of developmental e-cig exposure have focused on the neural and behavior outcomes in the offspring (*47*). Studies investigating different regions of the brain in mice have found that exposure to e-cig aerosols in early life can increase global DNA methylation of the brain (18 mg/ml; inhalation), alter the transcriptome of the frontal cortex (13-16 mg/ml; inhalation), and dysregulate gene expression in the hippocampus (13 mg/ml; inhalation) (*21, 22, 24*). Modulation of DNA methylation and the transcriptome of select brain regions suggests risk for chronic neuropathology later in life. Reports of reduced cognitive function and altered behavior patterns in adult offspring was reported in mouse models of early life e-cig exposure (18-24 mg/ml; inhalation) (*24, 48*). Craniofacial malformations have also been reported in an amphibian model of prenatal e-cig aerosol exposure (6-24 mg/ml; extracts), and the strong correlation between physical defects and neurological deficits implies that central nervous system development may also be impaired (*23*). Nicotine-free e-cig aerosol exposure during early life has been reported to induce increased adiposity in offspring with dysregulation of neuronal metabolic regulatory pathways (*19*). Many of these studies recorded neurological deficits after early life e-cig exposure even in the absence

of nicotine, further demonstrating the toxicity of e-cig aerosols on development and that e-cig use during pregnancy should be avoided.

1.7. Concluding Remarks

The popularity of e-cigs is increasing across all demographics, and especially among vulnerable populations such as teens and young adults (*9*). The lack of evidence in regard to e-cigs' perceived safety over other conventional tobacco products has raised great concern for public health. The effects of e-cig aerosols on pregnancy and early human development is currently unexplored due to the novelty of this tobacco product, thus, animal models are critical to revealing and understanding the outcomes of e-cig use during pregnancy (*49*). The reports outlined within this review offer great insight into the potential harm that exposure to e-cig aerosols during early life may have on offspring development. For these reasons e-cigs may not be as safe as previously believed and pregnant women should not be advised to use e-cigs or any tobacco product during pregnancy.

2. CHRONIC EXPOSURE TO E-CIG AEROSOLS DURING EARLY DEVELOPMENT CAUSES VASCULAR DYSFUNCTION AND OFFSPRING GROWTH DEFICITS*

2.1. Introduction

Electronic nicotine delivery systems (ENDS) have gained increasing popularity within the past few years (*50, 51*). ENDS are more commonly referred to as electronic cigarettes (e-cig) and using such devices has been given the term vaping. E-cig liquid is comprised of propylene glycol, glycerol, nicotine, and flavorings. These chemicals are aerosolized by a heating element within the e-cig and then inhaled/exhaled during vaping. Chemical analysis of e-cig aerosols has shown that a large number of hazardous chemicals are released while vaping, leading the Surgeon General of the United States to declare e-cig use as a public health concern (*2, 5, 52-54*). Advertisement of these products is largely geared towards younger demographics, in an effort to alter the perception of e-cigs as a safer or less-harmful alternative to traditional cigarette smoking (*55-58*). With e-cig use among adolescents and people of reproductive age increasing from 1.8% in 2010 to 25% in 2015, a closer look at the effects of vaping is imperative (*50, 51*). Since approximately half of women who smoke before pregnancy continue to

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* Reprinted with permission from "Chronic Exposure to E-Cig Aerosols during Early Development Causes Vascular Dysfunction and Offspring Growth Deficits" by Marcus R. Orzabal, Emilie R. Lunde-Young , Josue I. Ramirez, Selene Y. F. Howe, Vishal D. Naik, Jehoon Lee, Cristine L. Heaps, David W. Threadgill, and Jayanth Ramadoss, 2019. *Translational Research*, 207, 70-82, Copyright 2019 by Marcus Orzabal.

smoke during pregnancy and after delivery, it is crucial to assess the potential risks associated with developmental e-cig exposure during early life (*59, 60*).

Currently, there is a major knowledge gap in regard to the safety of e-cig use during pregnancy. To date, there are no human studies that report the health effects of gestational e-cig use. The few *in vivo* studies using animal models for e-cig vaping during pregnancy have found significant alterations to the pulmonary system of the mother and offspring, and to the central nervous system of offspring exposed during early life. Studies investigating the lungs of offspring exposed to e-cig vapor during pregnancy show dysregulation in gene expression associated with normal lung development (*20*). Neonatal e-cig exposure was reported to inhibit alveolar cell proliferation and postnatal lung development (*18*). In the developing fetal brain, gestational e-cig vaping was found to alter gene expression in the frontal cortex and result in localized inflammation of the hippocampus (*21, 22*). In many of these studies, it is suggested that constituents of the e-cig liquid other than nicotine may also play a role in the health effects associated with e-cig exposure in early life.

A common end-result of substance use in pregnancy is fetal growth restriction (*61, 62*). This teratogenic effect has been well documented in studies investigating the use of alcohol and traditional cigarettes during pregnancy (*1, 63, 64*). Further, alcohol drinking is frequently accompanied by smoking (*65, 66*). The growth deficits associated with substance abuse put offspring at increased risk for further health complications in later life (*67, 68*). Established models of intrauterine growth restriction (IUGR) demonstrate a decrease in maternal uterine artery blood flow (*69-71*). In normal

pregnancy, the uterine artery undergoes significant adaptations to accommodate the growing nutritional requirements of the fetus, with blood flow through the uterine artery increasing by approximately 30-50 fold by the third trimester (*72-75*). Disruption of these normal uterine vascular adaptations may be detrimental to proper fetal growth and development. Although e-cigs produce a large number of aerosols, their effects on fetal growth and postnatal development are practically unknown. Further, nothing is known about the effects of e-cig vaping on the maternal uterine artery (which delivers oxygen and nutrients to the feto-placental compartment), or the fetal umbilical vasculature.

With very few investigations on the effects of e-cig use during pregnancy, translational animal studies are both necessary and vital to understand the implications of gestational e-cig vaping on the developing fetus. To date, much of the animal research that aims to discern the correlation between vaping and maternal/infant health employs methods such as intraperitoneal injections, orogastric gavage, and unheated vaporization. These techniques lack the heating element and delivery method that is characteristic of human e-cig vaping. Since the metabolism, and thus the effects, of nicotine differ depending on the route of delivery, we utilized a highly translational method of e-cig aerosol delivery that allowed for a vaping topography common among current e-cig users (*76, 77*). Using our custom engineered vaping system in combination with our well-characterized pregnant rat model, we evaluated the effects of vaping e-cigs containing nicotine during gestation and exposure to these aerosols during early neonatal development on the overall growth of the offspring, and whether growth deficits are accompanied by altered maternal and fetal reproductive vascular hemodynamics.

2.2. Methods

2.2.1. Treatment Groups

All experimental procedures were in accordance with National Institutes of Health guidelines (NIH Publication No. 85-23, revised 1996), with approval by the Animal Care and Use Committee at Texas A&M University. Timed-pregnant Sprague-Dawley rats, 6-7 weeks old, were purchased from Charles River (Wilmington, MA) and housed in individual cages in a temperature-controlled room (23^{o}C) with a 12:12-hour light/dark cycle. Rats were randomly assigned to one of three treatment groups: Pair-Fed Control (Control), Pair-Fed Juice (Juice), and Juice + Nicotine (Nicotine). Prior to initiation of vaping, Control and Juice rats were yoked with a Nicotine vaping animal of similar weight, and the feed amount for these respectively yoked animals was matched daily, thus controlling for nutritional intake throughout the study. The Control group also served as a control for the vaping procedure. Control animals were maintained in vaping chambers identical to those in the Juice and Nicotine groups for an equivalent duration, but with only room air passing through the chamber at a flow rate matching the vaping groups. The Juice group controlled for any difference in the effects of vaping e-cig liquid in the absence of nicotine. The Nicotine group allowed for the testing of the effects of vaping e-cig liquid with nicotine, as a majority of e-cig consumers use devices containing e-cig liquid with nicotine.

2.2.2. E-cig Vaping System

A custom-engineered vaping chamber system with precision-controlled aerosol release technology was used to establish the e-cig vaping paradigm. This setup (Figure

2-1) allowed for uniform and simultaneous delivery of a customized e-cig vapor profile to all vaping treatment groups. We utilized a Sense Herakles Sub-Ohm Tank (Sense Technology Co., Ltd). The tank contains a replaceable kanthal (iron-chromium-aluminum alloy) 0.6 ohm dual vertical parallel coil, 4 channel adjustable airflow control, and was matched to a 60 W output source via the programmable atomizer. The apparatus included a programmable atomizer that produced e-cig vapor plumes and regulated the volume of liquid vaporized per unit time (Figure 2-2A). The programmable atomizer is compatible with a wide variety of e-cig vaping media and was used for all subjects within treatment groups. The atomizer used in our system to generate the aerosols was in line with the latest generation of e-cig atomizers currently available to and used by the public. The sophisticated software interface controlled voltage delivered to the programmable atomizer, puff duration, and puff frequency within the inhalation chamber. Airflow was directed throughout the system via silicone tubing (6 mm inner diameter, 10 mm outer diameter) and one-way exhaust valves to ensure unidirectional airflow. The inhalation chambers were airtight, amber, polymer containers resembling the animal housing cages. The software interface is unique in its ability to produce puff profiles identical to those produced by commercial e-cigs. All emissions from the inhalation chambers passed through activated charcoal filters to remove any harmful particles prior to passing through facility exhaust ducts.

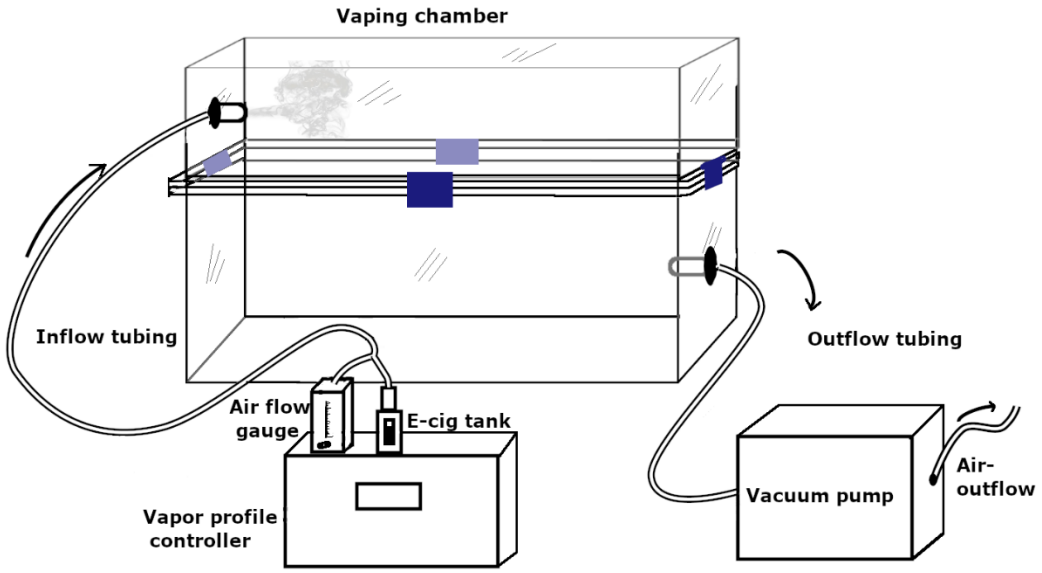

Figure 2-1 Schematic representation of e-cig vaping chamber apparatus. Reprinted with permission from (*34*).

2.2.3. Exposure Paradigm

Airflow through the chambers was kept at a constant flow rate of 2.5 L/min, with a one second e-cig vapor puff of approximately 42 mL puff volume dispensed every 20 seconds. The duration of the puff was held at one second to ensure proper ventilation of the chambers and removal of accumulating aerosols. To accommodate for the shorter puff duration, a higher power output and nicotine concentration were used since these parameters have been shown to effectively modulate nicotine yield during vaping sessions (*78*). The e-cig base liquid was compounded in-house at room temperature with a composition of 80:20 propylene glycol (Fisher) to glycerol (Acros Organics), similar to e-cig liquids bought in most vaping shops (*79*). This e-cig liquid ratio was preferred for

our paradigm to maintain adequate absorption of e-cig liquid by the cotton wick due to the relative viscosities of propylene glycol and glycerol. Recent studies on e-cig liquid composition have reported that propylene glycol based liquids provide a higher nicotine delivery ratio than glycerol based liquids (*80*). E-cig liquids used for the Nicotine group followed the same proportion guidelines as the e-cig base liquid with the addition of 5% (50 mg/mL) nicotine during acclimatization followed by 10% (100 mg/mL) nicotine. E-cig liquid nicotine concentration was selected with consideration for the average nicotine concentration of commercially available e-cig liquids (*76, 81*).

Our study utilized two different sets of animals for two different exposure periods to assess growth and cardiovascular effects of vaping during pregnancy and early development: 1. To address gestational effects, we utilized a prenatal-only exposure paradigm (Figure 2-2B), where dams underwent vaping treatment for three hours a day, five days a week, beginning on gestational day (GD) 5 until GD 20, two days prior to parturition (*21*); 2. To address maternal vaping after birth and resultant exposure to the aerosols in the environment during early postnatal life, we utilized a prenatal + postnatal paradigm (Figure 2-2C). Dams underwent vaping treatment for two hours a day, five days a week from GD 5 until GD 21, gave birth on GD 22, and then dams and pups resumed vaping on postnatal day (PND) 4 until PND 9, and were sacrificed on PND 10. The postnatal exposure paradigm beginning on PND 4 is an established paradigm in perinatal exposure models and was utilized to reduce imposed stress shortly after birth (*21, 22, 82-84*). For both prenatal and postnatal studies, the Nicotine group was first acclimatized to the vaping treatment during GD 5-8 utilizing a

lower dose of 5% nicotine in the e-cig liquid. Following the acclimatization period, the Nicotine group vaped 10% nicotine for the duration of the studies.

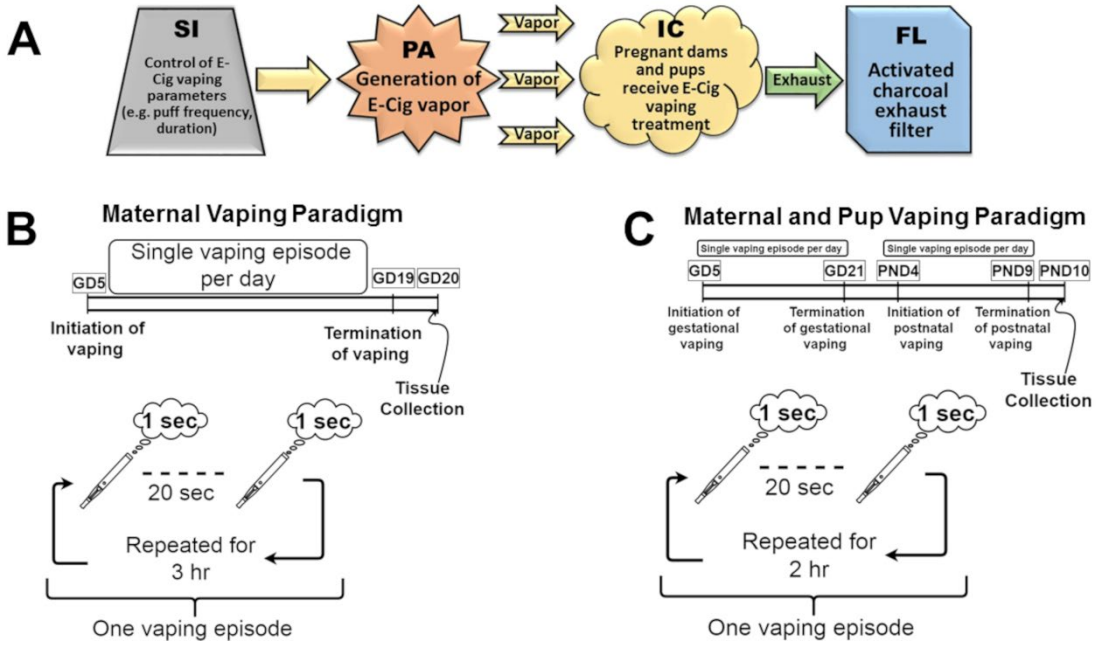

Figure 2-2 Flow chart depiction of e-cig aerosol production, exposure, and elimination. (A) The software interface (SI) controlled voltage delivered to the programmable atomizer (PA), which produced the e-cig vapor plume, as well as puff duration and frequency. The puffs traveled to the custom-engineered inhalation chamber (IC) and then through an activated charcoal filter (FL), which eliminated harmful emissions from the IC exhaust. (B) Maternal Vaping Paradigm: Pregnant dams underwent vaping for three hours, five days a week, from gestational day (GD) 5-19. A one second puff was dispensed every 20 seconds. (C) Maternal and pup Vaping Paradigm: Pregnant dams underwent vaping for 2 hours, five days a week, from GD 5-21. Dams gave birth on GD 22, and dams and pups underwent vaping treatment from postnatal day (PND) 4-10, for 2 hours a day. A one second puff was dispensed every 20 seconds. Reprinted with permission from (*34*).

2.2.4. Aerosol Analysis

Aerosol samples were collected in XAD-4 sorbent tubes (Sigma, Saint Louis, MO) at a flow rate of 1 L/min for 3 min. The aerosol was extracted through a sampling port on the side of the chamber via AirChek Touch sample pump (SKC, Houston, TX). Per operating instructions, pump airflow was calibrated prior to each collection using a chek-mate calibrator (SKC, Houston, TX). Capped tubes were stored at 4℃ in UV impermeable packaging until analysis. To identify chemical constituents, each sorbent tube was disassembled according to NIOSH 2551, desorbed in 1 mL modified ethyl acetate, and analyzed by GC/MS in triplicate. Agilent 7890B gas chromatograph (Agilent, Santa Clara, CA) and Agilent 5977A mass spectrometer (Agilent, Santa Clara, CA) were utilized for analysis (Health Research Inc., Roswell Park, Buffalo, NY). Chromatograph column properties included (HP-5): 30 m length, 0.32 mm inner diameter, 0.25 μm film, and 2 mm universal liner with wool. Sample volume of 1.0 μL was injected at 250℃. Helium was used as the carrier gas, at a constant flow rate of 1.7 mL/min. Oven temperature ranged from 110℃ to 250℃ (held one minute) at a rate of 10℃/min. Qualitative analysis of aerosols was carried out using National Institute of Standards and Technology (NIST) 14 Mass Spectral library and Flavors and Fragrances of Natural and Synthetic Compounds (FFNSC) 3 flavoring library (Health Research Inc., Roswell Park, Buffalo, NY).

2.2.5. Mass Spectrometric Analysis of Blood Nicotine Levels

A separate cohort of animals was utilized to assess blood nicotine concentration and were exposed to the same vaping paradigm administered for the different dependent measures (n=5). Blood samples were collected in 0.5 mL serum tubes (BD Biosciences) following the tail-bleed procedure outlined by Omaye et al. (*85*). Samples were collected on GD 11. Samples were collected just prior to initiation of the vaping exposure (time point = 0 hrs) and every 3 hrs after the start of the experiment up to 12 hrs, after which a 6 hr interval was used to obtain samples at time points 18 and 24 hrs. In one dam, one sample at 18 hrs could not be collected due to sampling complications. Samples were centrifuged at 10,000 g for 5 min at 4°C. Supernatant was removed and aliquoted into 100 uL portions, flash frozen in liquid nitrogen, and stored at -80°C until further processing.

Serum nicotine concentration was measured using liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). 20 µL of serum was mixed with 4 µL of 2.5 N NaOH and an extraction was performed by adding 120 µL of 50:50 methylene chloride: diethyl ether and stirring for 1.5 min. Samples were centrifuged at 4,000 rpm for 5 min and the organic phase was transferred to a 1.5 mL HPLC vial. Organic phase solvent was evaporated under a gentle stream of nitrogen gas at 35°C. Dried extract was reconstituted in 60 µL of deionized water. Liquid chromatography was performed at 30°C using a Varian diphenyl column (SN 285114); 50 mm long by 2 mm inner diameter. Particle size of stationary phase was 5 µm with an isocratic mobile phase

of 5% methanol in water (0.1% formic acid). An injection volume of 20 μL was used for LC-MS/MS analysis on an Agilent HPLC 1100.

2.2.6. Growth Measures

To assess the developmental implications of e-cig exposure on growth, body weight and crown-rump length were measured for all offspring. For the prenatal study, animals were sacrificed on GD 20, one day after the last vaping treatment, and fetal weight (number of dams, Control n=15; Juice n=11; Nicotine n=11) was recorded. Litter size between all groups in the prenatal cohort was not significantly different (average litter size, Control=11.87; Juice=12; Nicotine=11.18). Crown-rump length was recorded in fetuses from a subset of dams (number of dams, Control n=6; Juice n=5; Nicotine n=6); litter size between all groups was not significantly different (average litter size, Control=11.17; Juice=12.4; Nicotine=11.17). In the prenatal + postnatal study, a separate cohort of animals were utilized; litter size of each dam (number of dams, Control n=8; Juice n=9; Nicotine n=8) was culled to 8 to standardize nutrition for all pups across treatment groups. Animals were sacrificed on PND 10, one day after the last postnatal vaping treatment. Individual pup weight was collected at birth (PND 1) and daily from PND 3-10. Crown-rump length was measured for each pup on PND 10.

2.2.7. In Vivo Hemodynamic measurements

One day after the last vaping treatment and prior to sacrifice (GD 20), a subset of dams were imaged by Doppler/high-frequency ultrasonography to obtain heart rate and blood flow measurements (Control n=5, Juice n=5, Nicotine n=5). Ultrasound in combination with Doppler tracings is a noninvasive diagnostic tool that can be used to

measure maternal and fetal heart rate as well as flow through the specific reproductive vasculature. Animals were initially sedated using 5% isoflurane in oxygen (1 L/min) in an air-tight induction box for 2-4 mins before being moved to a heated table and fitted with a nose cone that provided 2% isoflurane in oxygen (0.50 L/min). The lower abdomen was shaved and ultrasonic transmission gel (EcoGel 100) was applied prior to probing. Measurements were acquired using a 40-MHz (MX550D) probe a Vevo® 3100 ultrasonograph (VisualSonics, Toronto, Canada). The maternal uterine artery and the fetal umbilical artery were imaged in both B-mode and color pulse wave Doppler to obtain measurement parameters. Vessel identification was established primarily by the unique waveform shape of the primary uterine artery, as well as relative position and structure within the animal (*86, 87*). A mean maximum velocity was calculated over three continuous cardiac cycles within the Doppler tracing of uterine and umbilical arteries. Transverse vessel diameter images were acquired in B-mode and were analyzed using specialized software (Vevo LAB, Fujifilm VisualSonics) to obtain accurate measurements. Uterine artery and umbilical artery flow rates were determined from the mean peak waveform velocity and the cross-sectional area of the respective vessel.

2.2.8. Statistics

Fetal weight and crown-rump length, pup weight and crown-rump length (PND 10), maternal/fetal heart rate, uterine artery blood flow, and umbilical blood flow were all analyzed by one-way ANOVA with treatment group as the sole independent variable. Postnatal growth measured from PND 1 to 10 was analyzed using a mixed ANOVA model with treatment group as the “between” factor and PND as the “within” factor.

Kruskal-Wallis One-Way Analysis of Variance on ranks was performed when needed (*88, 89*). When appropriate (when significance of a factor or of interaction was established by the initial analysis), an analysis of simple effect was performed for each postnatal day using one-way ANOVA as needed. Further, pair-wise comparisons were performed when appropriate using Tukey's test. All data are presented as mean $\pm$ SEM. The α level was established *a priori* at $P < 0.05$ for all analyses.

2.3. Results

2.3.1. Mass Spectrometric Analysis

Aerosolized compounds detected within the e-cig vaping chambers were analyzed by gas chromatography/mass spectrometry (Figure 2-3). All compounds listed were found in the front section of the sorbent tube; the back section was used to detect blow through (overloading of sorbent tube during aerosol sampling). Compounds detected in at least two of three runs/sample were considered present in the sample. Aerosolized chemicals providing only one hit during analysis were determined to be a product of noise. In addition to propylene glycol, glycerol, and nicotine, mass spectrometry analysis showed 17 other aerosols were detected in the aerosol samples. LC-MS/MS quantification of serum nicotine levels are represented by the median for each time point with 25% and 75% range in Figure 2-3 ($P=0.0085$, Kruskal-Wallis). Median serum nicotine concentration ranged from 7.30 to 27.69 ng/mL with a peak at 6 hours after the start of the exposure.

A **Mass Spectrometric Analysis of Aerosol Compounds in E-Cig Vaping Chamber**

Propylene Glycol
Glycerol (Glycerine)
Nicotine
Propanic Acid, Ethyl Ester
Isopropyl Acetate
n-Propyl Acetate
Formic Acid, Butyl Ester
Acetic Acid
Acetic Acid, Butyl Ester
Benzaldehyde, 2,4-dimethyl-
Nonane
Triethylamine
Butanal, 3-hydroxy-
Indan-5-ol
Pentanoic Acid, 5-hydroxy-, 2,4-di-t-butylphenyl Esters
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
5-Hexyn-3-ol
3,4-Hexanedione, 2,2,5-trimethyl
Isophthaladehyde
Oxalic Acid, Allyl Nonyl Ester

B

Time (Hr)	Serum Nicotine Concentration (ng/ml) Median and 25%-75% range
0	7.30 (6.63-8.72)
3	26.13 (16.48-115.40)
6	27.69 (16.65-98.04)
9	14.19 (10.00-77.48)
12	11.91 (8.94-38.55)
18	10.77 (7.85-15.47)
24	10.24 (8.55-12.50)

Figure 2-3 Mass spectrometric analysis of aerosol compounds and serum nicotine concentration. (A) The listed compounds met the detection criteria of being present in at least two of the three analysis runs per sample. (B) LC-MS/MS quantification of serum nicotine levels are represented by the median for each time point with 25% and 75% range. Reprinted with permission from (*34*).

2.3.2. Growth Measurements

Fetal and pup growth measurements are illustrated in Figure 2-4. There was no statistical difference in the maternal weight among the three treatment groups prior to the start of the study on GD 5 (Control, 208.7 ± 4.80 g; Juice, 209.5 ± 5.10 g; Nicotine, 217.1 ± 4.80 g), or on GD 20 (Control, 291.9 ± 11.00 g; Juice, 300.7 ± 11.40 g; Nicotine, 300.1 ± 11.40 g). Mean fetal weight in the Control group (2.33 ± 0.084 g) was not different from those in the Juice group (2.29 ± 0.078 g). The median fetal weight with 25% and 75% range for each group were $2.31_{(2.09\text{-}2.47)}$ g, $2.23_{(2.09\text{-}2.37)}$ g, and $1.39_{(1.13\text{-}1.63)}$ g in the Control, Juice, and Nicotine groups respectively. The Control and Juice groups were not different, and both these groups were significantly different from the Nicotine group ($P<0.001$, Kruskal-Wallis). Fetal weight in the Nicotine group (1.45 ± 0.16 g) was significantly decreased ($P < 0.0001$) by 46.56% compared to the Control group, and by 44.92% compared to the Juice group (Figure 2-4A). Fetal crown-rump length (Figure 2-4C) in the Control group (35.83 ± 0.44 mm) was not different from that in the Juice group (32.74 ± 1.96 mm). Fetal crown-rump length measured in the Nicotine group (27.29 ± 0.81 mm) was significantly decreased compared to the Control (↓23.83%; $P = 0.0002$) and Juice groups (↓16.65%; $P = 0.0129$). Average pup weight (PND 10; Figure 2-4B) in the Nicotine group (11.34 ± 1.06 g) was significantly decreased compared to the Control (19.04 ± 0.43 g; ↓40.44%; $P = 0.0002$) and Juice (19.12 ± 1.38 g; ↓40.69%; $P < 0.0001$) groups. Nicotine pup weights were significantly decreased ($P < 0.05$) on PND 4 to 10 when compared to the Control and Juice groups. There was no difference in pup weight between the Control and Juice groups during PND 1 to 10 (Figure 2-4E).

Pup crown-rump length (PND 10; Figure 2-4D) in the Nicotine group (49.67 ± 1.25 mm) was significantly decreased ($P < 0.0001$) compared to the Control (65.97 ± 1.42 mm; ↓24.71%) and Juice (62.80 ± 1.81 mm; ↓20.91%) groups.

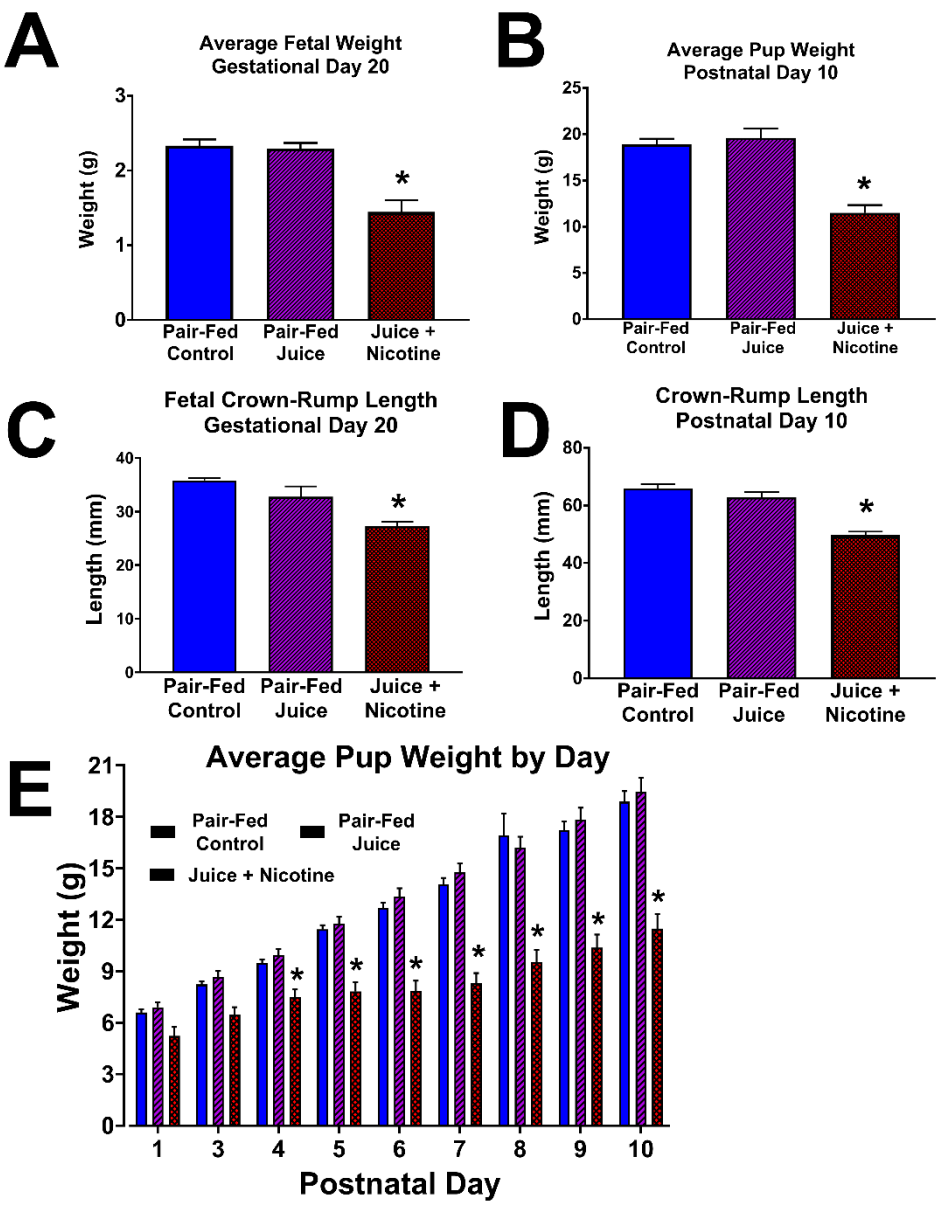

Figure 2-4 Effects of e-cig vaping during early development on offspring growth. Following gestational and postnatal vaping, fetal and pup weight and crown-rump length were measured on gestational day (GD) 20 and postnatal day (PND) 10, respectively, one day after the last vaping episode. (A) Mean fetal weight in the Juice + Nicotine group was decreased compared with Pair-Fed Control and Pair-Fed Juice groups ($P < 0.0001$). (B) Pup weight in the Juice + Nicotine group was decreased compared with both Pair-Fed Control ($P = 0.0002$) and Pair-Fed Juice groups ($P < 0.0001$). (C) Fetal crown-rump length in the Juice + Nicotine group was decreased compared to the Pair-Fed Control and Pair-Fed Juice groups ($P = 0.0002$, $P = 0.0129$, respectively). (D) Following prenatal + postnatal vaping, crown-rump length in the Juice + Nicotine group

was decreased compared with that in the Pair-Fed Control and Pair-Fed Juice groups. ($P < 0.0001$). (E) Postnatal pup weight in the Juice + Nicotine group was decreased compared with the Pair-Fed Control and the Pair-Fed Juice Groups on PND 4-10. Values are mean + SEM, * indicates statistical significance, $P < 0.05$. Reprinted with permission from (*34*).

2.3.3. Hemodynamic Measurements

There was no significant difference in the maternal heart rate (Figure 2-5A) or fetal heart rate (Figure 2-5B) among the three treatment groups. These values were determined using Doppler tracing by measuring the elapsed time required for three waveforms to cycle (peak-to-peak). There was no significant difference in the maternal uterine artery blood flow or the fetal umbilical artery blood flow between the Control and Juice group. Uterine artery and umbilical artery blood flow were significantly decreased in the Nicotine group compared to the Control group (uterine artery, ↓49.50%, $P = 0.0489$, Figure 2-5C; umbilical artery, ↓65.33%, $P = 0.0164$, Figure 2-5D).

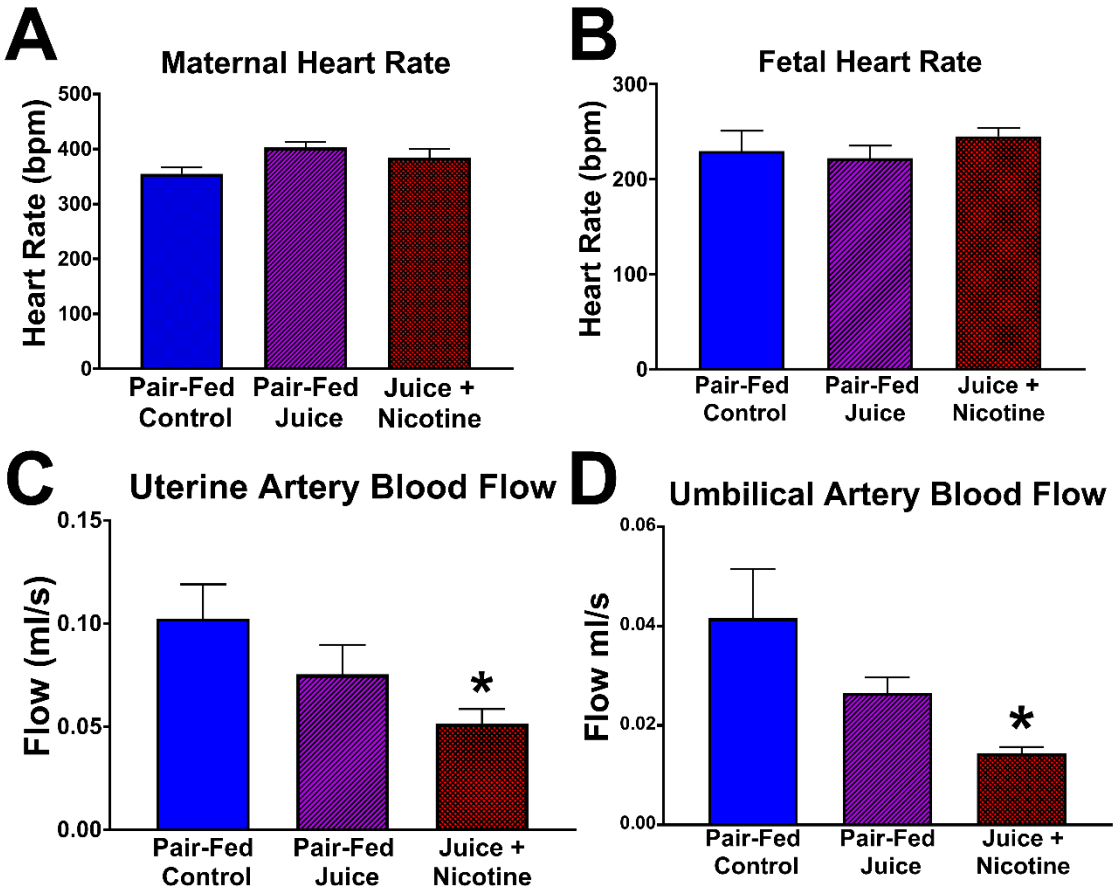

Figure 2-5 Effect of gestational e-cig vaping on maternal uterine and fetal umbilical artery hemodynamics. On GD 20, (A) maternal heart rate and (B) fetal heart rate were not different following chronic e-cig vaping during pregnancy. However, in the Juice + Nicotine group, both (C) maternal uterine artery blood flow ($P = 0.0489$) and (D) fetal umbilical artery blood flow ($P = 0.0164$) were reduced compared with those in the Pair-Fed Control. Values are mean + SEM, * indicates statistical significance, $P < 0.05$. Reprinted with permission from (*34*).

2.4. Discussion

To our knowledge, this is the first study to assess health effects of e-cig use during pregnancy and early development in a rat model using the latest generation e-cig atomizer technology. The vaping chamber system allowed for a vaping topography

directly comparable to current e-cig users. From this study, we can glean three important conclusions: 1. Chronic exposure to e-cig aerosols containing nicotine during early development can have deleterious health effects on the exposed offspring; 2. Vaping e-cigs containing nicotine during pregnancy leads to a reduction in offspring weight and crown-rump length, consistent with an IUGR phenotype (*70, 90, 91*); and 3. Blood flow is markedly decreased in both the maternal uterine artery and fetal umbilical artery (a strong indicator of early-onset IUGR) after vaping e-cigs containing nicotine during pregnancy (*92*). Our data collectively demonstrate that chronic vaping of e-cigs containing nicotine during pregnancy can lead to potentially harmful effects in the developing fetus.

Overall, our data show that chronic exposure to e-cig aerosols containing nicotine during early development can have deleterious health effects on the exposed offspring. It is well known that smoking traditional cigarettes during pregnancy is harmful to the developing fetus, largely because the combustion process produces toxic substances, such as carbon monoxide (*93*). E-cigs have gained increasing popularity within the past few years, particularly among those in the reproductive age demographic (*10*). One reason for this is that users openly perceive these devices as a safer alternative to traditional cigarette smoking, as e-cigs aerosolize a liquid comprised of propylene glycol, glycerol, nicotine, and flavorings rather than burning tobacco (*55-58*). Since e-cigs are a recent development, it may take many years for research to fully delineate possible risks associated with their use. A common limitation among current e-cig related research that complicates the evaluation of health effects attributed to e-cig use is

in part due to the wide selection of different e-cig products and modification packages with unique settings available to the public. Currently, little is known regarding e-cig safety, especially health effects during pregnancy. A recent survey reported that without accounting for the adverse effects of nicotine, 45% of pregnant women believed e-cigs were less harmful than traditional cigarettes (*10, 94*). If pregnant women use e-cigs that contain nicotine as a harm reduction alternative, our data show that this exposure could potentially result in injury to the developing fetus. Serum nicotine levels described in this study closely reflect the concentration of nicotine found in the blood of active e-cig users and traditional cigarette smokers (*3, 95-99*). Experienced e-cig users (many may be ex-cigarette smokers) maintain an almost identical average blood nicotine concertation when compared to tobacco users (*3, 100*). Daily baseline concentration of serum nicotine in individuals who use tobacco products is known to vary from person to person depending on extent of use and rate of clearance (*76, 101*). Traditional cigarette smokers self-titrate nicotine by controlling the number and frequency of cigarettes smoked per day in order to achieve a baseline blood nicotine concentration (*96*). The daily concentration of blood nicotine in habitual smokers typically reaches a steady state of approximately 20-50 ng/mL, but can vary within the range of 5-100 ng/mL (*95-98*). While nicotine can accumulate in the blood throughout the day the rate of clearance is high enough that differences in nicotine absorption from one day to the next are negligible (*102*). However, the continual slow release of nicotine deposited in various body tissues results in serum nicotine levels above 0 ng/mL even after a brief period of abstinence (*103*). To date, few studies have been performed on the effects of vaping e-

cigs during pregnancy and/or early development. In support of our findings, it has been shown in other animal model studies, vaping e-cigs containing nicotine during pregnancy altered gene expression in the pulmonary system and produced central nervous system (CNS) dysregulation in the offspring (*20*). For instance, in the lung, alveolar cell proliferation and postnatal lung development were inhibited by neonatal e-cig exposure (*18*). In the CNS, gestational e-cig vaping altered gene expression in the frontal cortex and resulted in localized inflammation of the hippocampus (*21, 22*). Collectively, we and others demonstrate that use of e-cigs containing nicotine during pregnancy is not as safe as is often perceived, with multiple organ systems being influenced by e-cig exposure.

Our data show chronic exposure to nicotine from e-cigs during early development results in a dramatic decrease in both fetal and pup weights as well as their crown-rump lengths. This is a significant finding because a smaller than average weight at birth is typically accompanied by other severe complications such as respiratory distress syndrome and necrotizing enterocolitis, making growth restriction an important clinical indicator of perinatal morbidity. (*92, 104, 105*). Developmental growth restriction is also associated with an increased risk for developmental delays, infant mortality, and manifestation of chronic diseases later in life (*67, 68*). Thus, growth restriction resulting from chronic exposure to e-cigs containing nicotine during development may increase risks for a myriad of complications at birth and for developing chronic disease in adulthood. Nutrient delivery to the fetus has been shown to be a critical factor influencing fetal growth and development (*105*). Substance abuse,

alcohol consumption, and tobacco use during pregnancy frequently lead to IUGR as a result of poor nutrient delivery (*1, 61-63*). Further, vaping e-cigs containing nicotine may directly inhibit acetylcholine-facilitated transport systems responsible for moving vital amino acids across the placenta (*106*). To control for nutritional intake in the nicotine-exposed animals, we included pair-fed control groups, and found no difference in the maternal weights at the end of the exposure paradigm. Therefore, we conjectured that nicotine vaping-induced fetal growth restriction may still result from decreased nutrient delivery from the mother to the feto-placental compartment via reduced blood flow in the maternal uterine artery. Although we did not measure maternal and fetal oxygen and nutrient concentrations in this study, we tested if there is a decrease in blood flow in the maternal uterine artery, which directly controls the supply of oxygen and nutrients to the feto-placental compartment (*75, 107*).

A common theme in IUGR animal models is reduced nutrient delivery resulting from a lower than normal blood flow in the uterine artery (*70-72, 75*). Our study is the first to investigate the effects of e-cig exposure during pregnancy on both the uterine artery and umbilical cord hemodynamics. Utilizing ultrasonography paired with real-time Doppler tracings, we were able to determine blood flow through specific reproductive vasculature. The maternal uterine artery undergoes profound adaptations to accommodate a 30-50 fold increase in gestational blood flow to the developing fetus which are crucial for sustaining growth and normal fetal development (*72-75*). Our data showed that blood flow through both the maternal uterine artery and the fetal umbilical artery in animals exposed to e-cigs containing nicotine were 49.50% and 65.33% lower

than those in the control group, respectively. Nicotine has been shown to possess strong vasoconstrictor properties, and acts by stimulating sympathetic outflow and impairing endothelial-dependent vasorelaxation, effectively increasing the vascular resistance in the feto-placental compartment (*108, 109*). A lower blood volume per unit time delivered to the feto-placental compartment will proportionally and dramatically decrease the amount of nutrients and oxygen delivered. The current study is not capable of dissecting if the decreased uterine artery blood flow leads to growth restriction, or, alternatively, if e-cig vaping-induced growth restriction leads to a decreased demand for oxygen and nutrients from the mother. As far as the fetal compartment is concerned, a decreased blood flow in the umbilical circuit may be directly due to a lower cardiac output in the growth-restricted fetuses, or an increase in nicotine-induced resistance offered by the placental blood vessels, which are in series with the umbilical artery and the umbilical vein (*92, 110, 111*). This study provides evidence that vaping nicotine during pregnancy can produce potentially harmful effects for the developing fetus by altering the vascular adaptations necessary for normal pregnancy. Although a cause and effect relationship cannot be established in our current study design, future studies are warranted to mechanistically test if growth restriction can be produced by directly inhibiting the uterine blood flow similar to the decreases seen in this study.

We utilized the Juice treatment group in this study to control for any difference in the effects of e-cig vaping in the absence of nicotine. Our data indicate that constituents in the e-cig aerosol other than nicotine did not produce growth restriction or impact the assessed maternal and fetal hemodynamics following gestational e-cig

vaping, however, it is important to note that there may be other underlying health effects of these aerosols that have yet to be determined. Although a large body of prenatal animal research demonstrates that nicotine alone can result in significant disruptions to normal development spanning nearly all major organ systems, it remains unclear whether nicotine is the single component contributing to negative health outcomes observed in offspring exposed to e-cig aerosols during early life. The vapor created by e-cigs is a result of heating the e-cig liquid at high temperatures. This heating process can alter the chemical profile of the original liquid to produce a large number of hazardous aldehydes and numerous other chemical byproducts (*5, 52, 112*). Reactive aldehydes, such as those in Figure 2-3, have been shown to negatively impact maternal and fetal health across multiple organ systems (*113, 114*). Many of the chemicals produced by e-cigs have been observed to be potentially dangerous if ingested, yet the effects of these chemicals and their byproducts when inhaled is not fully understood. The analysis of aerosols described in this study will require further quantitative analysis to identify chemicals of particular investigative interest. Several studies support the idea that chemicals other than nicotine play a role in altering normal development with evidence that e-cig liquid alone can effect neurodevelopment and metabolic function of offspring (*21, 69*). Thus, it is imperative that further research be done to investigate the impact that such chemicals have on development. Flavorings used in commercial e-cig liquids further complicate investigations into the health effects of e-cig vaping due to their highly variable recipes and inconsistent manufacturing procedures (*6, 11*). Flavoring

components were not included in this study, however, they may play an integral role in the health outcomes associated with e-cig vaping.

Further research is urgently warranted to fully understand the largely unknown health consequences regarding e-cig safety during pregnancy. The effects of nicotine have the potential to encompass all aspects of fetal development. To fully evaluate the effects of developmental e-cig aerosol exposure on offspring growth, a comprehensive examination of developmental parameters beyond those reported herein must be explored. Although our study shows that vaping nicotine is harmful to early development, constituents of the e-cig liquid other than nicotine may exacerbate the negative outcomes associated with e-cig exposure (*115, 116*). Since alcohol consumption during pregnancy is usually compounded by tobacco product use, investigating the combined effects of alcohol consumption and e-cig vaping during pregnancy is also necessary (*65, 66*). Additionally, it is imperative that e-cig flavorings be evaluated for safety, as recent studies show these may contain harmful additives, such as diacetyl, that can significantly influence negative health outcomes (*117-119*). Lastly, temporal studies are necessary to determine the various developmental windows of vulnerability for specific organ systems.

3. IMPACT OF GESTATIONAL ELECTRONIC CIGARETTE VAPING ON AMINO ACID SIGNATURE PROFILE IN THE PREGNANT MOTHER AND THE FETUS*

3.1. Introduction

Exposure to tobacco products during pregnancy is known to have a host of detrimental effects on maternal and fetal health, yet an estimated 65% of current U.S. smokers continue to smoke throughout pregnancy (*120*). Consumption of electronic cigarettes (e-cigs), one of the latest forms of tobacco products, has increased rapidly over the past decade and has become a popular choice among youth and young adults according to current Center for Disease Control evaluations (*2, 121*). E-cigs are tobacco products that were originally intended to serve as a cessation and harm reduction tool for traditional cigarette smokers. E-cigs come in a multitude of shapes and sizes, however, all e-cigs operate following a similar set-up, of a battery-powered handheld device that rapidly heats an e-cig liquid (usually containing nicotine and flavorings) to produce an aerosol that is inhaled or "vaped" by the user. In spite of a rise in the popularity of e-cigs, there are few studies examining their effects on human physiology and development. Due to the novelty of e-cig vaping, there are neither long-term studies in humans, nor studies on the effects of e-cigs on human pregnancy. Furthermore, there is only a small set of studies examining the short-term effects of e-cig vaping in adult

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humans. The limited number of animal studies conducted on the effects of e-cig aerosol exposure during pregnancy have established that e-cigs can negatively affect fetal growth, the cardiopulmonary system, and nervous system development (*25, 122, 123*). The large knowledge gap on the effects of e-cig vaping necessitates a systematic investigation into how these devices impact pregnancy and development. In order to elucidate potential molecular mechanisms underlying e-cig-induced gestational adaptations, we assessed the concentration of 22 amino acids (AAs) in maternal and fetal compartments that are critical for optimal growth and normal fetal development.

AAs are the basic building blocks for many biological molecules and are involved in a number of functions essential for survival, including protein synthesis/degradation, DNA/RNA synthesis, immune response, and metabolic regulation (*124, 125*). These compounds are vital to all living organisms, and their concentrations must be maintained to sustain homeostasis (*126*). During pregnancy, AAs are transported to the rapidly growing fetus via the placenta (*127*). While the fetus is capable of synthesizing some AAs on its own, a large portion of AAs are obtained from maternal circulation (*124*). In normal pregnancy, the concentration of AAs in fetal plasma is typically higher than maternal plasma, indicating active transport, but certain pathologies can alter AA transfer across the placenta and consumption of AAs by the fetus (*128, 129*). Supplementation of specific AAs during pregnancy and lactation has been shown to ameliorate intrauterine growth restriction (IUGR), reduced skeletal muscle mass, and oxidative stress in rats and pigs (*130, 131*). In humans and animal models, exposure to traditional tobacco smoke results in altered placental morphology that is directly

correlated with reduced concentrations of several AAs in maternal and fetal compartments (*132, 133*).

Furthermore, the fetal lungs are an especially sensitive target of prenatal exposure to tobacco products, with strong evidence showing that tobacco exposure in humans can result in reduced respiratory function/capacity, induction of asthma, and development of chronic obstructive pulmonary diseases later in life (*1, 64*). There is minimal knowledge on the role of AAs in fetal lung development outside the scope of protein synthesis and there are no current examinations on the effects of tobacco products, or nicotine, on the AA profile within the lung. With the invention and rise in the use of e-cigs, the need for investigation has become imperative to assess the effects of prenatal e-cig aerosol exposure on AA concentration in the maternal and fetal plasma, as well as in developing fetal lungs. We hypothesized that prenatal exposure to e-cig aerosols would have a direct impact on the signature profile of the 22 major AAs in maternal and fetal plasma, as well as in male and female fetal lungs of late gestation rats, and thus help identify critical molecular pathways underlying vaping-associated pathologies during development.

3.2. Methods

3.2.1. Treatment Groups

All experimental procedures were in accordance with National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996), with approval by the Animal Care and Use Committee at Texas A&M University. Timed pregnant Sprague-Dawley rats were purchased from Charles River (Wilmington, MA), and housed in a

temperature-controlled room at 23°C with a 12:12-hr light/dark cycle. All rats were breed at 6-8 weeks of age (~200g body weight; first and only pregnancy). Rats were randomly assigned to one of three treatment groups which included a pair-fed control (CTRL) group exposed to room air; a pair-fed group exposed to e-cig aerosols without nicotine (EC-Base); and a group exposed to e-cig aerosols containing nicotine (EC-Nic). Prior to the start of treatment, dams in CTRL and EC-Base groups were yoked to a dam of similar weight in the EC-Nic group. Diet administered to both pair-fed groups was matched to the daily amount of feed consumed by dams in the corresponding EC-Nic group to control for nutritional effects of e-cig vaping on pregnancy. Pair-fed treatment groups have been previously shown to adequately control for nutritional intake in a prenatal exposure model (*134*). In addition to being a nutritional control, the CTRL group served as a control for exposure to e-cig aerosols and for the overall vaping treatment procedure. During the exposure paradigm, CTRL dams were placed in e-cig vaping chambers identical to the chambers used for e-cig vaping treatment. CTRL dams were maintained in these chambers for the same time duration as EC-Base and EC-Nic groups, with only room air flowing through the chamber. The EC-Base group allowed for the identification of differential effects due to e-cig aerosol exposure in the absence of nicotine, however, the majority of human e-cig consumers use vaping devices that contain nicotine. To account for this, the EC-Nic group reflects a physiologically relevant e-cig aerosol exposure with nicotine.

3.2.2. Vaping Treatment

The E-cig vaping treatment was conducted using a custom engineered e-cig aerosol exposure system that allowed for the simultaneous and discreet delivery of either e-cig aerosols or room air to specific chambers as previously described (*34*). The binge e-cig vaping paradigm utilized in this study has been shown to produce serum nicotine levels (median peak serum nicotine concentration equal to 27.7 ng/mL), comparable to moderate/high level human smokers and resembles human e-cig vaping topography (*34, 76, 135, 136*). Additionally, the chemical constituents of the aerosols produced by the vaping chamber system were found to resemble the chemical profile of aerosols derived from human e-cig vaping devices (*34, 137, 138*). Dams were exposed to the vaping treatment for 3 hours per day, 5 days per week from gestation day (GD) 5-20 (*22, 34, 122*). Each episode of vaping treatment utilized a commercially available e-cig atomizer (Sense Herakles) that produced a 1 sec puff of ~42 mL every 20 seconds. The e-cig base liquid utilized for the EC-Base group was compounded in-lab with an 80:20 composition ratio of propylene glycol (Fischer) and glycerol (Fischer), respectively. E-cig liquid utilized for the EC-Nic group maintained the same proportional guidelines as the base liquid with the addition of either 5% (50 mg/mL) nicotine during acclimatization or 10% (100 mg/mL) nicotine. During an acclimatization period from GD 5-8, the EC-Nic dams were exposed to e-cig aerosols produced using the 5% nicotine e-cig liquid. Following the acclimatization period, the EC-Nic dams were exposed to e-cig aerosols produced using the 10% nicotine e-cig liquid for the remainder of the exposure paradigm.

3.2.3. Tissue Collection

All groups were sacrificed on GD 21, one day after the last vaping treatment. This study did not contain a humane endpoint prior to date of termination. Growth parameters were collected at the time of euthanasia: maternal weight, fetal weight, fetal crown rump length, and placental weight. Placental weight was used to calculate placental efficiency, as the ratio of fetal body weight to placental weight. Maternal and fetal blood samples were also collected at the time of euthanasia. Dams were quickly euthanized and a hysterectomy was performed to remove the fetuses. Growth parameters were recorded for all fetuses prior to removal of whole lungs from one male and one female per dam. All tissue samples were flash frozen in liquid nitrogen and stored at -80°C until further processing.

3.2.4. Amino Acid Analysis

The AA profiles of maternal and fetal plasma samples were determined by HPLC analysis following standard procedures (*139, 140*). Briefly, 0.5 mL of sample was added to a 12x75 mm polypropylene tube and mixed via vortex with 0.5 mL of 1.5 M $HClO_4$ and 0.25 mL of 2 M K_2CO_3. After centrifugation of the tube at 3,000 g for 15 min the supernatant was collected and used for HPLC analysis. For the determination of AA profile in fetal lung tissues, a portion of tissue (~100 mg) was homogenized in 1 ml of 2 M $HClO_4$ (perchloric acid) and rinsed with 1 mL HPLC-grade water. The homogenate was neutralized with 0.5 ml of 2 M K_2CO_3. The whole solution was centrifuged at 3,000 g for 10 min, and the supernatant fluid was analyzed for free AAs using HPLC methods (*139, 141*). Concentrations of AAs in samples were quantified based on

authentic standards from Sigma Chemicals (St. Louis, MO, USA), using the Waters Millenium-32 workstation (*142, 143*).

3.2.5. Calculations

The unit of analysis was equal to the dam or litter for each group. All groups had n=6 dams for a total of 18 animals. All animals were included in the analysis. Threshold for statistical significance was determined *a priori* as $P < 0.05$. Maternal weight, fetal weight, fetal crown rump length, and placental efficiency were analyzed using one-way ANOVA with treatment group as the independent variable. Concentrations of individual AAs in the maternal plasma, fetal plasma, and fetal lungs were also analyzed using one-way ANOVA with treatment group as the independent variable.

3.3. Results

3.3.1. Growth Parameters

Pregnancy related growth measures are depicted in Figure 3-1. Maternal weight on GD 21 was not significantly different among the three treatment groups. Fetal weight in the EC-Nic group was found to be significantly decreased ($P < 0.0001$) compared to both the CTRL (↓36.7%) and EC-Base (↓35.4%) groups. Fetal crown rump length in the EC-Nic group was significantly decreased ($P < 0.0001$) compared to both CTRL (↓16.6%) and EC-Base (↓15.4%) groups. Placental weight (not shown) in EC-Nic group was significantly decreased ($P = 0.0014$) compared to both CTRL (↓35.6%) and EC-Base (↓31.6%) groups, however, placental efficiency (fetal weight/placental weight) was not significantly different among treatment groups.

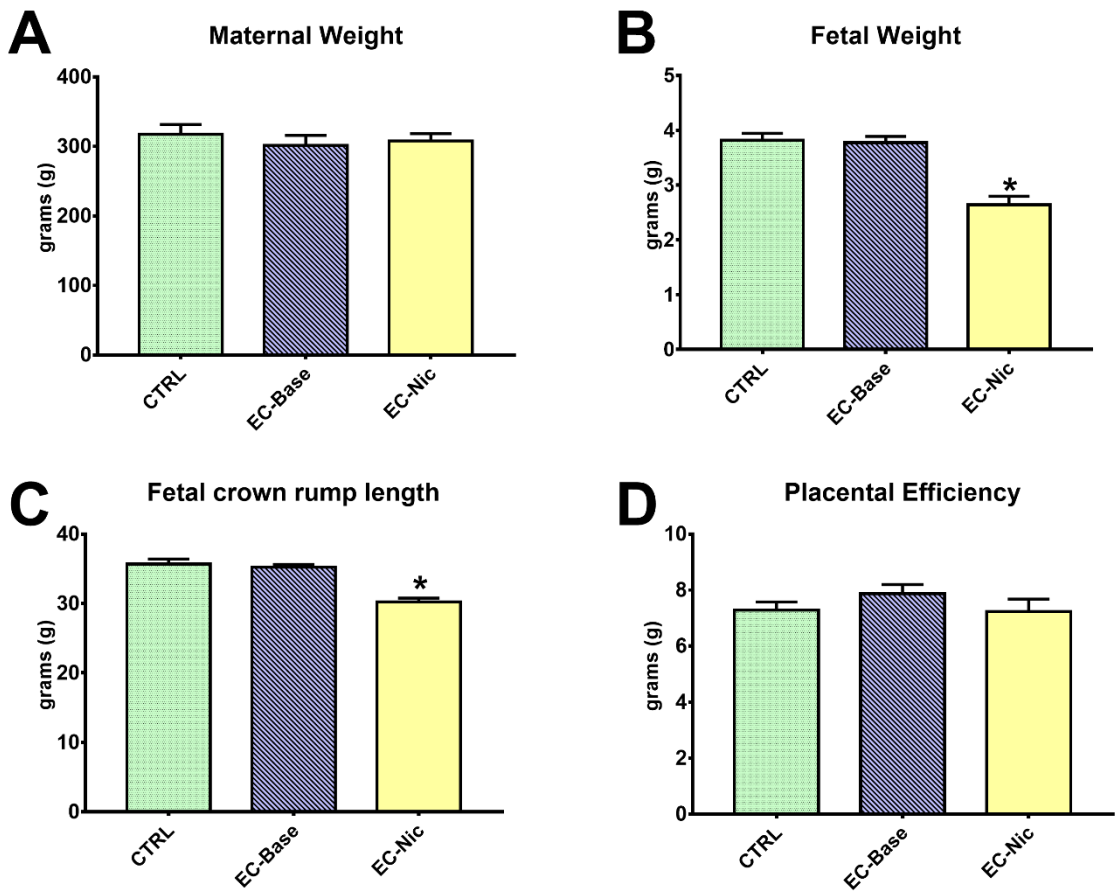

Figure 3-1 Effect of prenatal e-cig aerosol exposure on maternal and fetal growth on gestational day 21. Placental efficiency was calculated as ratio of placental weight to fetal weight. *Indicates significant difference compared to Control; $P < 0.05$. Reprinted with permission from (*144*).

3.3.2. Amino Acid Concentrations

Concentrations of AA, in maternal and fetal plasma were determined to examine the amount of free AAs present in the plasma during pregnancy in both maternal and fetal circulation. The concentrations of AAs in maternal plasma of the EC-Base and CTRL groups were not significantly different. The maternal plasma of the EC-Nic group

showed a significant increase in the concentration of 13 AAs compared to those in the CTRL group (Figure 3-2). The AAs altered in maternal plasma of the EC-Nic group compared to CTRL were the following: serine ($P = 0.0201$), glutamine ($P = 0.0002$), histidine ($P = 0.0002$), glycine ($P = 0.0144$), threonine ($P = 0.0062$), citrulline ($P = 0.0005$), arginine ($P = 0.0064$), tyrosine ($P = 0.0027$), tryptophan ($P = 0.0071$), valine ($P = 0.0063$), phenylalanine ($P < 0.0001$), leucine ($P = 0.0083$), and ornithine ($P < 0.0001$). The maternal plasma of the EC-Nic group also showed significant differences in the concentrations of 14 AAs compared to those in the EC-Base group (Figure 3-2). The AAs altered in maternal plasma of the EC-Nic group compared to EC-Base group were the following: asparagine ($P = 0.0459$), serine ($P = 0.0197$), glutamine ($P = 0.0003$), histidine ($P = 0.0001$), threonine ($P = 0.0151$), citrulline ($P = 0.0008$), arginine ($P = 0.0022$), tyrosine ($P = 0.0035$), tryptophan ($P = 0.0071$), valine ($P = 0.0088$), phenylalanine ($P = 0.0004$), isoleucine ($P = 0.0426$), leucine ($P = 0.0086$), and ornithine ($P = 0.0001$).

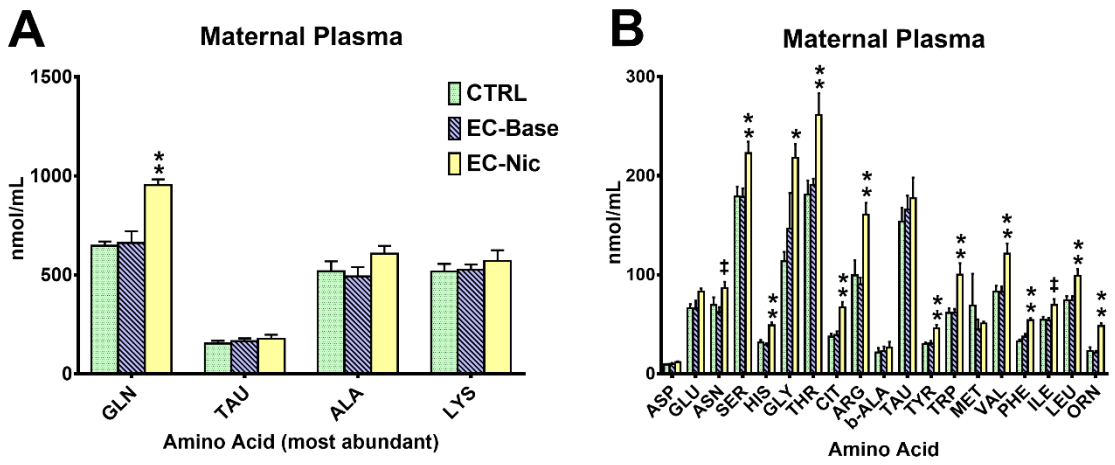

Figure 3-2 Effect of prenatal e-cig aerosol exposure on maternal plasma amino acid (AA) concentrations. The AAs altered in maternal plasma of EC-Nic group compared to CTRL are: serine (↑), glutamine (↑), histidine (↑), glycine (↑), threonine (↑), citrulline (↑), arginine (↑), tyrosine (↑), tryptophan (↑), valine (↑), phenylalanine (↑), leucine (↑), and ornithine (↑). The AAs altered in maternal plasma of EC-Nic group compared to EC-Base group are: asparagine (↑), serine (↑), glutamine (↑), histidine (↑), threonine (↑), citrulline (↑), arginine (↑), tyrosine (↑), tryptophan (↑), valine (↑), phenylalanine (↑), isoleucine (↑), leucine (↑), and ornithine (↑). The concentration of AAs in maternal plasma of the EC-Base and CTRL groups were not significantly different. *Indicates significant difference compared to Control; **Indicates significant difference compared to Control and EC-Base; ‡Indicates significant difference compared to EC-Base only; $P < 0.05$. Reprinted with permission from (*144*).

The concentrations of AAs in the fetal plasma of the EC-Base and CTRL groups were not significantly different. The fetal plasma of the EC-Nic group showed a significant increase in the concentration of three AAs compared to the CTRL group (Figure 3-3). The AAs altered in the fetal plasma of the EC-Nic group compared to CTRL were the following: glutamate ($P = 0.0421$), phenylalanine ($P = 0.0066$), and ornithine ($P = 0.0039$). The fetal plasma of EC-Nic group also showed significant differences in the concentration of three AAs compared to those in the EC-Base group.

The AAs altered in the fetal plasma of the EC-Nic group compared to EC-Base group were the following: glutamine (P = 0.0372), arginine (P = 0.0038), and ornithine (P = 0.0018).

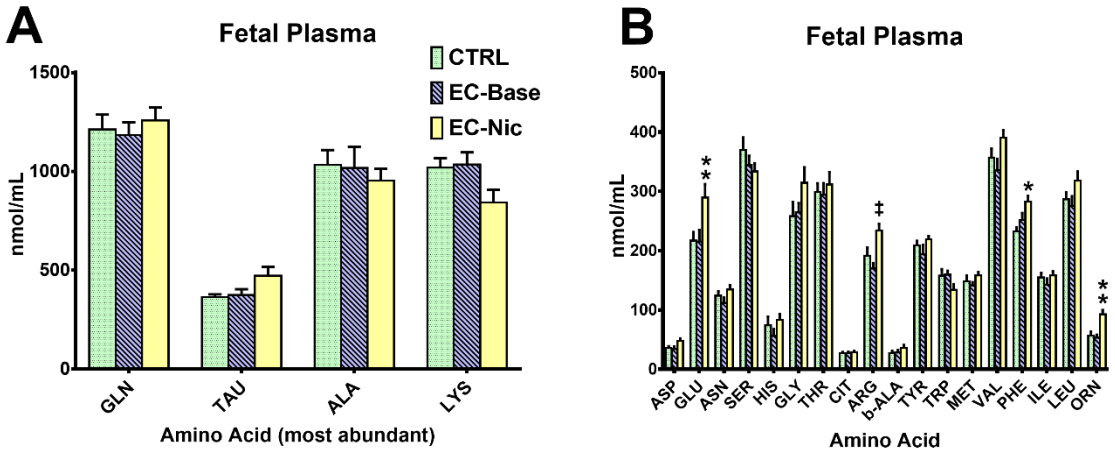

Figure 3-3 Effect of prenatal e-cig aerosol exposure on fetal plasma amino acid (AA) concentrations. The AAs altered in the fetal plasma of the EC-Nic group compared to CTRL are: glutamate (↑), phenylalanine (↑), and ornithine (↑). The AAs altered in the fetal plasma of EC-Nic group compared to EC-Base group are: glutamine (↑), arginine (↑), and ornithine (↑). The concentration of AAs in fetal plasma of the EC-Base and CTRL groups were not significantly different. *Indicates significant difference compared to Control; **Indicates significant difference compared to Control and EC-Base; ‡Indicates significant difference compared to EC-Base only; $P < 0.05$. Reprinted with permission from (*144*).

Concentrations of AA, in male and female fetal lungs from each group, were compared to determine the accumulation or deficit of AAs in tissues targeted by prenatal tobacco product exposure and to establish sex-linked effects of e-cig aerosols on the developing respiratory system. The male fetal lungs of EC-Nic group showed a

significant increase in the concentration of 11 AAs compared to those in the CTRL group (Figure 3-4). The AAs altered in the male fetal lungs of EC-Nic group compared to CTRL were the following: aspartate (P = 0.0226), glutamate (P = 0.0016), asparagine (P = 0.0071), threonine (P = 0.0018), citrulline (P = 0.0142), arginine (P = 0.0114), methionine (P = 0.0456), valine (P = 0.0019), isoleucine (P = 0.0074), leucine (P = 0.0167), and ornithine (P = 0.0007). The only AA found to be significantly different in male fetal lungs of EC-Nic compared to EC-Base group was ornithine (P = 0.0003). The male fetal lungs of EC-Base group showed a significant difference in the concentration of threonine (P = 0.0144) compared to CTRL group, whereas concentrations of glutamate (P = 0.0560), citrulline (P = 0.0999), arginine (P = 0.0755), and valine (P = 0.0605) trended to be different compared to CTRL group.

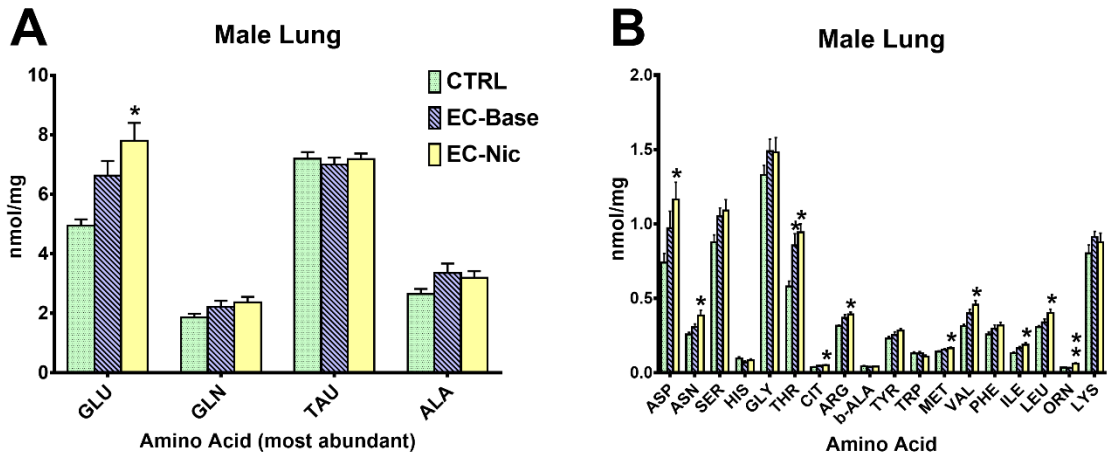

Figure 3-4 Effect of prenatal e-cig aerosol exposure on male fetal lung amino acid (AA) concentrations. The AAs altered in the male fetal lungs of EC-Nic group compared to CTRL are: aspartate (↑), glutamate (↑), asparagine (↑), threonine (↑), citrulline (↑), arginine (↑), methionine (↑), valine (↑), isoleucine (↑), leucine (↑), and

ornithine (↑). The only AA found to be significantly different in male fetal lungs of EC-Nic compared to EC-Base group was ornithine (↑). The male fetal lungs of EC-Base group showed a significant difference in the concentration of threonine (↑) compared to CTRL group. *Indicates significant difference compared to Control; **Indicates significant difference compared to Control and EC-Base; $P < 0.05$. Reprinted with permission from (*144*).

The signature of AAs that were impacted in the male and female fetal lungs of the EC-Nic group compared to CTRL shared a number of similarities. The female fetal lungs of EC-Nic group showed a significant difference in the concentration of 9 AAs compared to the CTRL group (Figure 3-5). The AAs altered in the female fetal lungs of EC-Nic group compared to those in the CTRL were the following: aspartate ($P = 0.0455$), glutamate ($P = 0.0045$), asparagine ($P = 0.0052$), glutamine ($P = 0.0395$), threonine ($P = 0.0047$), citrulline ($P = 0.0403$), valine ($P = 0.0088$), isoleucine ($P = 0.0355$), and ornithine ($P = 0.0021$). The only AA found to be significantly different in female fetal lungs of EC-Nic group compared to EC-Base group was ornithine ($P = 0.0098$). The female fetal lungs of EC-Base group showed a significant difference in the concentration of threonine ($P = 0.0145$) and alanine ($P = 0.0449$) compared to those in the CTRL group, whereas the concentrations of citrulline ($P = 0.0956$) and arginine ($P = 0.0964$) trended to be different compared to those in the CTRL group. A similar pattern in the identity of dysregulated AAs in the male and female fetal lungs suggests that there are minimal sex-linked effects contributing to prenatal e-cig aerosol induced alterations to AA signature profile, in the developing lungs.

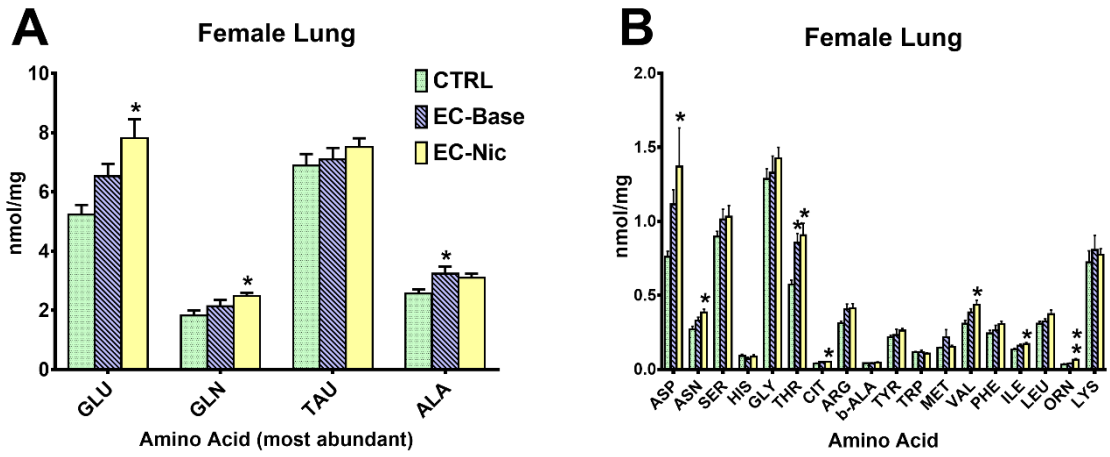

Figure 3-5 Effect of prenatal e-cig aerosol exposure on female fetal lung amino acid (AA) concentrations. The AAs altered in the female fetal lungs of EC-Nic group compared to CTRL are: aspartate (↑), glutamate (↑), asparagine (↑), glutamine (↑), threonine (↑), citrulline (↑), valine (↑), isoleucine (↑), and ornithine (↑). The only AA found to be significantly different in female fetal lungs of EC-Nic compared to EC-Base group was ornithine (↑). The female fetal lungs of EC-Base group showed a significant difference in the concentration of threonine (↑) and alanine (↑) compared to CTRL group. *Indicates significant difference compared to Control; **Indicates significant difference compared to Control and EC-Base; $P < 0.05$. Reprinted with permission from (*144*).

3.4. Discussion

AAs play an integral role in a number of physiological processes, including regulation of oxidative stress, cell signaling, protein synthesis, acid-base balance, and synthesis of small molecules such as nitric oxide (*124, 125*). The present study examined AA concentrations in maternal and fetal plasma as well as fetal lung tissue using HPLC analyses to determine the impact of prenatal e-cig aerosols on the AA signature profile during late pregnancy. To our knowledge, the data presented herein are the first to show the impact of prenatal e-cig aerosol exposure on AA concentrations in the maternal and

fetal compartments, and is the first study to examine the effects of tobacco products on the AA profile of the developing fetal lung. These novel findings reveal valuable information pertaining to the effects of e-cig aerosol vaping: 1) exposure to e-cig aerosols with nicotine during pregnancy alters the AA profile in maternal and fetal plasma, but e-cig aerosols without nicotine do not; 2) e-cig aerosols with and without nicotine alter the AA profile in both male and female fetal lungs; 3) sex has a minimal effect on the pattern of dysregulation of AAs in the fetal lungs; 4) exposure to e-cig aerosols containing nicotine increased the concentration of ornithine in all major tissues that were analyzed; and 5) patterns of AA dysregulation in fetal lungs of the EC-Nic group may indicate altered nitric oxide production, induced by e-cig aerosol exposure.

Exposure to tobacco products and nicotine during pregnancy is known to produce IUGR in human and animal models (*14, 145*). Rodent models of prenatal nicotine exposure have been critical to understanding the altered physiology of pregnancy as it relates to human development. While no animal model is perfectly analogous to humans, pregnancy induced vascular adaptations and pulmonary development are well established in rodent models (*146-148*). We previously demonstrated that our model of prenatal exposure to e-cig aerosols containing nicotine produces significantly reduced fetal and postnatal growth, which is accompanied by a reduction in blood flow in the maternal uterine artery and fetal umbilical artery (*34*). Of the 22 AAs measured in this study, the concentrations of more than half were found to be dysregulated in the plasma of dams exposed to e-cig aerosols containing nicotine, when compared to EC-Base and CTRL groups. Nicotine appears to be the main

influencing factor on AA signature profile alterations in the mother, since there were no significant differences between the EC-Base and CTRL maternal plasma. Analyses of fetal plasma revealed a smaller number (three) of AAs that were dysregulated by e-cig aerosol exposure with nicotine. Similar to maternal plasma, there were no differences in AA concentrations in fetal plasma between EC-Base and CTRL groups. In normal pregnancy, there is a significant correlation between maternal and fetal plasma AA concentrations (*149*). Early studies in humans have shown that IUGR is correlated to a significant increase in the concentration of maternal plasma AAs, yet fetal plasma concentrations are reduced; these studies may partially explain the alterations reported herein (*129, 150*). In contrast, we found that the fetal plasma of EC-Nic group showed a significant increase in the concentration of several AAs that may be attributed to a decreased catabolism of amino acids (especially glycine), a decrease in protein synthesis, an increase in protein degradation, or their combination, leading to reduced fetal protein synthesis in a growth-restricted fetus. An increase in the circulating level of glycine (a precursor of glutathione [a major antioxidant peptide]) may be an adaptation response of the dam to oxidative stress.

The rate of AA transport across the placental barrier is determined by hormonal regulation, solute concentration gradients, and the abundance and availability of binding sites of specific transport proteins within the placental tissue (*151-153*). Although these transport proteins were not quantified in our model, nicotine is known to reduce the transfer of AAs across the placenta by inhibiting active and facilitated transport (*154-156*). Thus, despite a significant increase in the concentrations of maternal plasma AAs,

many of these increases may not be reflected in fetal plasma due, in part, to decreased transport across the placenta and/or decreased fetal protein synthesis. The combined effects of IUGR and placental exposure to nicotine may potentially produce a pattern of increases in AA concentration in the maternal plasma that does not directly correlate to the pattern of AA concentration dysregulation expressed in the fetal plasma.

Previous studies on the effects of tobacco products and nicotine exposure during pregnancy has labeled the fetal lungs as a susceptible target of developmental dysregulation, which may result in lifelong complications such as asthma and the development of chronic obstructive pulmonary disease (*45, 157, 158*). There is very little data examining the AA profile of fetal lungs, and there are no current evaluations on the effects of prenatal e-cig aerosol exposure on fetal lung AAs. In this study, exposure to e-cig aerosols with and without nicotine had a significant effect on the concentrations of several key groups of AAs in the fetal lungs. In fetal lungs exposed to e-cig aerosols with nicotine, there was significant dysregulation in 11 of the 22 AAs in males and 9 of the 22 AAs in females, with nearly complete overlap in the identity of altered AAs between the two sexes. In male fetal lungs, the concentrations of arginine, methionine, and leucine were significantly different in the EC-Nic group compared to CTRL, but were not different in the female lungs. In the female lungs of EC-Nic group, the concentration of glutamine was significantly different compared to CTRL, but was not different in male fetal lungs. The only AA to be altered in both male and female lungs of EC-Nic and EC-Base groups compared to CTRL was threonine. In the female lungs of EC-Base group, there was also a significant difference in the concentration of alanine

compared to CTRL. In the EC-Base group there was a trend towards significant difference in the concentrations of glutamine, citrulline, arginine, and valine in the male fetal lungs, and a trend towards significant difference in citrulline and arginine in the female fetal lungs compared to CTRL group. Patterns of AA dysregulation in the male and female fetal lungs of the EC-Nic group does not suggest that sex plays a major role in the dysregulation of the AA profile in fetal lungs exposed to prenatal e-cig aerosols. An increase in the concentration of branched-chain amino acids (BCAA – valine, leucine, and isoleucine) in the fetal lungs of the EC-Nic group may indicate protein degradation, insulin resistance, and a potential source of inflammation (*159, 160*). *In vitro* studies that examined the effects of the presence of exogenous BCAA on mouse endothelial cells, proposed that BCAA results in the activation of mTORC1 which modulates the production of reactive oxygen species, inflammatory gene expression, and leukocyte adhesion (*161*). Importantly, there were significant differences in AA concentrations in both the EC-Nic and EC-Base group, which indicates that chemical constituents other than nicotine in the e-cig aerosols do have an effect on the lungs and may contribute to altered fetal development supporting the claim that fetal lungs are susceptible to developmental dysregulation induced by prenatal exposure to tobacco products like e-cigs.

Exposure to e-cig aerosols containing nicotine may also contribute to altered pulmonary development by disrupting nitric oxide (NO) production. NO is a major signaling molecule that contributes to a large number of physiological pathways and is known to mediate several aspects of pulmonary development (*162, 163*). In fetal rat lung

explants, branching morphogenesis of airways was increased by the addition of a NO donor up to a certain concentration, with higher concentrations of NO resulting in diminished airway branching, demonstrating a need for strict NO regulation in fetal lung development (*164*). NO is generated through the conversion of arginine to citrulline, catalyzed by nitric oxide synthase (NOS) (*165, 166*). Previous studies have shown that the supplementation of citrulline in neonatal rats, and subsequent increase in NO production, ameliorates reduced alveolar growth and pulmonary hypertension in a model of O_2-induced bronchopulmonary dysplasia (*167*). NOS activity can be inhibited by the presence of arginase II, which is responsible for the conversion of arginine to ornithine in the urea cycle and competes for arginine as a substrate (*168*). Incidentally, ornithine was the only AA that was significantly increased in all tissue types of the EC-Nic group compared to EC-Base and CTRL groups. Increased concentrations of citrulline, aspartate, arginine, and ornithine in the fetal lungs may indicate an e-cig-induced redirecting of arginine from the NO synthesis pathway to the urea cycle. The sequestering of arginine supply from the NO synthesis pathway to the urea cycle, as documented in several experiments and cell types, is accompanied by reduced NO production (*169, 170*). Without sufficient NO, the fetal lungs may not be able to develop normally and may result in prenatal e-cig aerosol-induced respiratory pathologies in neonatal and adult life.

3.5. Perspectives

The data herein are novel for offering a glimpse into the relevant molecular alterations potentially contributing to prenatal e-cig aerosol-induced disruption to

pregnancy, in both the mother and the fetus. This study was limited to the analysis of free AAs in the tissues examined, therefore, future investigations are needed to expand on the mechanisms underlying e-cig aerosol-induced alterations to the AA signature profile during pregnancy. AAs are the base unit of proteins and are crucial for a number of biological pathways, especially during pregnancy. Although e-cigs are used as a harm-reduction tool for traditional tobacco smokers, there is growing evidence that e-cig aerosols with and without nicotine can have damaging effects on the physiology of pregnancy and development. The data obtained from this study provides additional support that gestational e-cig aerosol exposure can impact crucial biological processes and exemplifies the need for extensive research on exposure to e-cig aerosols during pregnancy.

4. IMPACT OF E-CIG AEROSOL VAPING ON FETAL AND NEONATAL RESPIRATORY DEVELOPMENT AND FUNCTION

4.1. Introduction

Electronic cigarette (e-cig) use during pregnancy has become a major health concern in recent years and is perpetuated by the perception that e-cigs are less harmful than traditional combustible cigarettes (*2, 171, 172*). Global estimates reveal that over half (53%) of daily smokers will continue to smoke throughout pregnancy with implications that e-cig users follow a similar trend (*8*). Although the claim that these devices are less harmful than traditional cigarettes is largely unsubstantiated due to lack of safety studies, the United States Surgeon General warns that exposure to tobacco products, like e-cigs, during pregnancy may result in damaging and life-long consequences for the offspring (*1*). E-cigs come in a multitude of shapes and sizes which typically includes a handheld battery that rapidly heats a metal coil to aerosolize an e-cig liquid. E-cig liquids primarily consist of propylene glycol, glycerin/glycerol, nicotine, and flavorings. All compounds are approved for oral consumption by the FDA in limited amounts, albeit without heating to high temperatures as occurs in e-cigs; however, the inhalation of these chemicals in high concentrations is relatively unexplored (*173*). Consequently, it is important to investigate the impact of e-cig aerosol exposure on pregnancy and to identify pathways of e-cig aerosol-induced disruptions to normal development.

The teratogenic effects of nicotine are well established, but there is a large knowledge gap on the effects of nicotine exposure during pregnancy in the context of e-cigs. There has only been one study evaluating birthweight in humans, which determined that exposure to prenatal e-cig aerosols increases the risk for a low birthweight pregnancy (*174*). Of the few studies investigating the effects of prenatal e-cig exposure in animal models, most are focused on the neurological outcomes due to the potent neurotoxicity of nicotine (*16*). Researchers have found that gestational exposure to the chemical constituents of e-cigs in mice results in dysregulation of the frontal cortex transcriptome (*21*), localized inflammation of the hippocampus (*22*), and altered memory and behavior of adult offspring (*24, 48*). In addition to the neurobiological effects, e-cigs have been shown to alter the development of several other organ systems in murine models (*25*).

The developing fetal lungs have been identified as a sensitive target organ for prenatal tobacco product exposure (*157*). Lung development is a lengthy process that begins *in utero* and continues throughout the early stages of neonatal life, which increases the chances of altered development due to environmental toxicants and poses a risk for the development of respiratory diseases later in life (*175*). Early studies in mice on the effects of e-cig exposure during the first 10 days of life demonstrate impaired lung growth and reduced alveolar development (*18*). Gestational exposure to e-cig aerosols has been shown to induce an altered inflammatory environment and increased DNA methylation of adult lungs (*20*). The *Wnt* signaling pathway, which is crucial for proper organogenesis of the lung, has been reported to be altered by prenatal e-cig

exposure and was associated with structural malformation of the lung (*123*). Prenatal e-cig aerosols were also shown to dysregulate the extracellular matrix of lung parenchyma in a sex dependent manner (*122*). The limited information regarding gestational e-cig exposure on pulmonary development and offspring respiratory outcomes is consistent with the idea that the developing lungs are a sensitive target organ for tobacco product exposure and indicates a need for a more thorough examination.

In this study, we investigated the impact of gestational e-cig aerosol exposure on offspring pulmonary development through a multi-level approach (Figure 4-1). At the cellular level, we assessed the effect of e-cig aerosols on the fetal lung transcriptome to identify potential molecular pathway targets. At the tissue-specific level, we examined how e-cig aerosols effects the histological structure, organization, and dimensions of the neonatal lung. At the organ function level, we assessed the impact of e-cigs aerosols on the pulmonary mechanics of the offspring to understand how developmental disruptions influence the function of the respiratory system. Thus, we hypothesized that prenatal exposure to e-cig aerosols with and without nicotine will significantly dysregulate gene expression in the developing lungs, alter lung structure with reduced alveolar proliferation, and restrict offspring pulmonary mechanics.

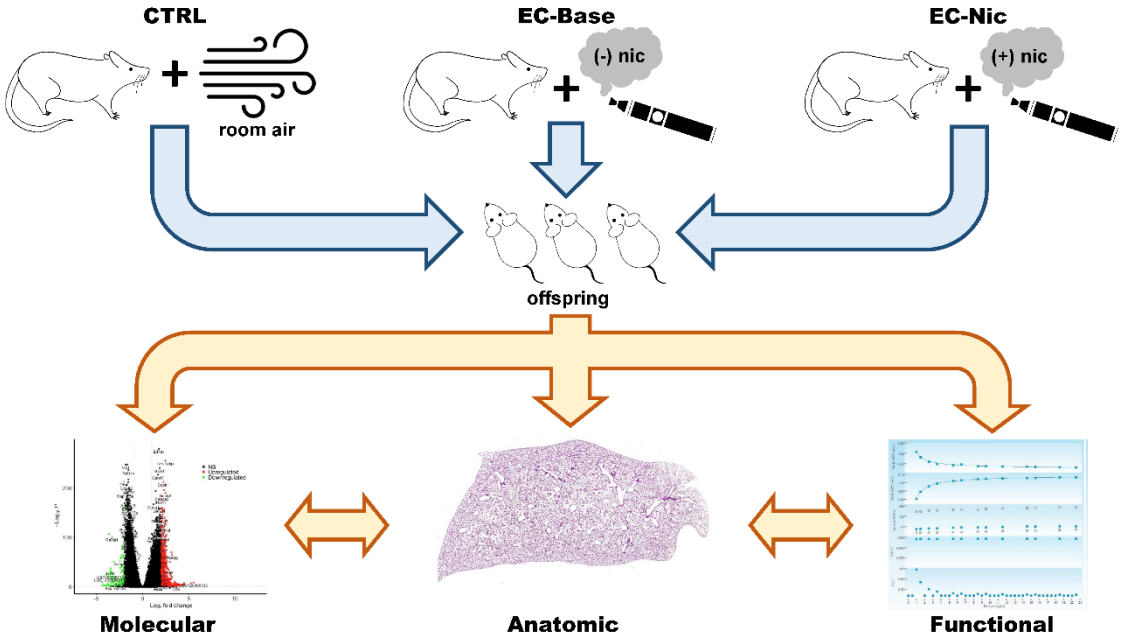

Figure 4-1 E-cig aerosol-induced alterations to fetal and neonatal respiratory development. Three-pronged approach to investigate impact of prenatal e-cig aerosol exposure on fetal and neonatal respiratory development and function. CTRL = room air control; EC-Base = e-cig aerosol vaping without nicotine; EC-Nic = e-cig aerosol vaping with nicotine.

4.2. Methods

4.2.1. Treatment Groups

All experimental procedures were in accordance with National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996), with approval by the Animal Care and Use Committee at Texas A&M University. Timed pregnant Sprague-Dawley rats, approximately 6 weeks of age, were purchased from Charles River (Wilmington, MA), and housed in a temperature-controlled room at 23°C with a 12:12-hr light/dark cycle. Dams were randomly assigned to one of the three treatment groups: a

pair-fed control (CTRL) group exposed to room air; a pair-fed group exposed to e-cig aerosol vaping without nicotine (EC-Base); or a group exposed to e-cig aerosol vaping containing nicotine (EC-Nic). Prior to the start of treatment, dams in CTRL and EC-Base groups were yoked to a dam of similar weight in the EC-Nic group. Diet administered to both pair-fed groups was matched to the daily amount of feed consumed by dams in the corresponding EC-Nic group to account for any nutritional effects of e-cig vaping on pregnancy. The CTRL group also served as a control for exposure to e-cig aerosols and for the overall vaping treatment procedure. During the exposure paradigm, CTRL dams were placed in e-cig vaping chambers identical to the chambers used for e-cig vaping treatment. CTRL dams were exposed to room-air only for the same time duration as EC-Base and EC-Nic groups. The EC-Base group allowed for the identification of differential effects due to e-cig aerosol exposure in the absence of nicotine.

4.2.2. Vaping Exposure Paradigm

The E-cig vaping treatment was conducted using a custom engineered e-cig aerosol exposure system that allowed for the simultaneous and discreet delivery of either e-cig aerosols or room-air to specific chambers as previously described (*34*). The binge e-cig vaping paradigm utilized in this study has been shown to produce serum nicotine levels (median peak serum nicotine concentration = 27.7 ng/mL), comparable to moderate/high level human smokers and resembles human e-cig vaping topography (*34, 59, 76, 95*). Additionally, the chemical constituents of the aerosols produced by the vaping chamber system were found to resemble the chemical profile of aerosols derived from human e-cig vaping devices (*52, 78, 79, 81*). Dams were exposed to the vaping

treatment for 3 hours per day, 5 days per week from gestation day (GD) 5-20 (*21, 22, 34, 122*). Each episode of vaping treatment utilized a commercially available e-cig atomizer (Sense Herakles/Zues Sub Ohm) that produced a 1 sec puff of ~42 mL every 20 seconds. E-cig base liquid used for the EC-Base group was compounded in-lab with an 80:20 composition ratio of propylene glycol (Fischer) and glycerol (Fischer), respectively. E-cig liquid utilized for the EC-Nic group maintained the same proportional guidelines as the base liquid with the addition of either 5% (50 mg/mL) nicotine during acclimatization or 10% (100 mg/mL) nicotine. During an acclimatization period from GD 5-8, the EC-Nic dams were exposed to e-cig aerosols produced using the 5% nicotine e-cig liquid. Following the acclimatization period, the EC-Nic dams were exposed to e-cig aerosols produced using the 10% nicotine e-cig liquid for the remainder of the exposure paradigm.

4.2.3. Growth Assessment

To assess the impact of e-cig exposure on offspring growth, body weight and crown-rump length were measured on GD 21 (n=6 for all groups) and PND 4 (n=5 for all groups). Neonatal weight was also recorded on postnatal day (PND) 10 (n=6 for all groups). Maternal weight was recorded on GD 21 prior to euthanasia (n=5 for all groups). Dams were quickly euthanized on GD 21, one day after the last e-cig exposure, and a hysterectomy was performed to remove the fetuses. Growth parameters were recorded for all fetuses prior to removal of whole lungs from one male fetus per dam. Neonatal growth measures were collected from one male pup per dam.

4.2.4. RNA Sequencing and Molecular Targets Assessment

Dams were sacrificed on GD 21 and fetuses were removed via hysterectomy. Whole lungs were collected from one male fetus per dam (n=6 for all groups). All tissue samples were flash frozen in liquid nitrogen and stored at -80°C until further processing. Samples were homogenized using TRIzol, and total RNA was isolated according to Invitrogen protocol. The quality of RNA was assessed using the Agilent TapeStation RNA assay. RNA was quantified by Qubit Fluorometric assay. All samples were normalized to the same starting concentration. Sequencing libraries were prepared using the TruSeq Stranded mRNA Library Prep kit from Illumina, with each sample uniquely indexed to allow for pooling of all samples in a single sequencing run. Library size and quality were assessed using the Agilent TapeStation D1000 DNA assay. Samples were normalized to approximately 4 nM and run simultaneously. Sequencing was performed using an Illumina NovaSeq 6000 S4 XP paired-end 150 cycle sequencing run.

The RNA-seq libraries were assessed for quality using FastQC v0.11.9 and trimmed for adapters and low-quality bases using Cutadapt version 3.0 (*176*). The resulting reads were mapped to the mRatBN7.2 Rattus norvegicus genome using HISAT2 version 2.2.1 (*177*). Differential expression analyses were conducted in R using DESeq2 (*178*), followed by functional enrichment analysis with gprofiler2 0.2.0 (*179*) and pathway analysis using GAGE 2.40.2 (*180*).

4.2.5. Lung anatomic Assessment and Histology

Whole lungs were randomly collected from one male pup per dam on PND 4 (n = 4-5) via installation fixation with 10% formalin at 25 mm water (*181*). All lungs were

placed in 10% formalin overnight and maintained in 70% ethanol solution until further processing. Weight and volume of fixed neonatal lungs were recorded. Volume was determined using Archimedes' principle of water displacement as previously described (*181*). Fixed tissues were embedded into paraffin blocks and cut longitudinally at 5 μm thickness along the posterior coronal plane. Sections were collected every 10 steps following a systematic uniform random sampling procedure to ensure sampling occurs at multiple depths throughout the lung (*182*). Six sections per animal were collected for all groups. All slides were stained utilizing standard hematoxylin and eosin staining procedures.

Digital images of each slide were created using bright field light microscopy combined with cellSens software (Olympus) at 4x magnification. Sections were assessed by a researcher blinded to the identity and group of each sample. ImageJ was utilized to collect morphological measurements, including mean linear intercept (MLI) and radial alveolar counts (RAC). An ImageJ plugin designed specifically to obtain MLI was used (*183*). Briefly, 10 non-overlapping regions of 1000x1000 pixels were selected for each sample. Images were overlaid with a set of 15 partially transparent test lines (chords). Individual chords crossing alveolar septa, isolated based on pixel color, were identified and MLI was calculated as the distance between intercepts. To calculate RAC, ImageJ was used to insert a test line perpendicular to the center of a distal bronchiole to the nearest parenchyma or lung pleural surface. The number of septa that intersect the test line were recorded. RAC was measured for five whole lung sections per sample as previously described (*184*). A minimum of 25 test lines were examined for each sample.

4.2.6. Neonatal Lung Mechanics

One male pup per dam was anesthetized with an intraperitoneal injection of 30 mg/kg pentobarbital and tracheotomized with a 16 gauge cannula on PND 10 (n=6 for all groups). Animals were connected via cannula to a FlexiVent (SCIREQ, Montreal, Quebec, Canada) and injected with 0.8 mg/kg of pancuronium to halt spontaneous breathing. Animals were placed on a heating pad at 39°C and mechanically ventilated for 3-5 min at 150 breath/min until starting measurements. Deep inflation of the lungs to a pressure of 30 mmH_2O was utilized to determine when spontaneous breathing ceased. Three inflation perturbations were used to assess lung mechanics: deep inflation, snapshot-150, and Quick Prime-3 (*185*). Deep inflation was used to calculate inspiratory capacity and serve as a recruitment maneuver. Snapshot-150 was used to calculate dynamic respiratory resistance (Rrs) and elastance (Ers). Quick prime-3 was used to calculate airway resistance (Rn), tissue damping (G), and tissue elastance (H). Partial pressure-volume (PV) loops were used to calculate static compliance (Cst) and area of the curve. All perturbations were run sequentially and automatically through the FlexiVent software, and were repeated three times per subject. Animals were euthanized immediately after the experiment was completed by an overdose of pentobarbital (200 mg/kg).

4.2.7. Data Analysis

The unit of analysis was equal to the dam or litter for each group. A total of 36 animals were utilized for these studies, 18 for fetal measurements and tissues, and 18 for neonatal measurements and tissues. Sigma plot software (Systat) was used to run all

statistical analyses. Threshold for statistical significance was determined *a priori* as $P < 0.05$, trends were determined to be $0.0.5 < P > 0.1$. Maternal and offspring growth measures, as well as placental efficiency, lung weight and lung volume were analyzed using one-way ANOVA with treatment group as the sole independent variable. Mean linear intercept, radial alveolar counts, and pulmonary mechanics parameters were also analyzed using one-way ANOVA with treatment group as the sole independent variable.

4.3. Results

4.3.1. Effect of E-Cig Aerosol Vaping on Fetal and Neonatal Growth

Offspring growth is a strong predictor of developmental deficiencies and adult-onset diseases (*70, 186*). To assess the impact of prenatal e-cig aerosol exposure on fetal and neonatal growth, we recorded weight and crown-rump length (CRL) on gestational day (GD) 21 and postnatal day (PND) 4 (Figure 4-2), as well as weight on PND 10. Maternal weight was not significantly different among the three groups (CTRL = room air control; EC-Base = e-cig aerosol vaping without nicotine; EC-Nic = e-cig aerosol vaping with nicotine) on GD 21, one day after the last vaping exposure and one day before delivery (EC-Nic=298±15.0 g; EC-Base=322±12.1 g; CTRL=302.8±14.2 g). Fetal weight in the EC-Nic group (2.90±0.31 g) was significantly decreased compared to CTRL (3.87±0.32 g; $P = 0.024$) and EC-Base (3.86±0.17 g; $P = 0.025$) groups. Fetal CRL in the EC-Nic group (30.57±1.40 mm) was also significantly decreased compared to CTRL (34.82±1.35 mm; $P = 0.019$) and EC-Base (34.83±0.41 mm; $P = 0.019$) groups. Fetal weight and CRL of the EC-Base group were not different compared to CTRL group. Placental efficiency, calculated as a ratio of fetal body weight to placental weight,

was not found to be different across the three groups (EC-Nic=8.45; EC-Base=7.76; CTRL=8.14).

Neonatal weight on PND 4 was significantly decreased in the EC-Nic group (7.06±0.55 g) compared to CTRL (10.31±0.53 g; P = 0.001) and EC-Base (10.90±0.78 g; P = 0.003) groups. Neonatal CRL on PND 4 was also significantly decreased in the EC-Nic group (42.39±1.79 g) compared to CTRL (48.45±1.02 g; P = 0.002) and EC-Base (49.67±0.85 g; P = 0.006) groups. Neonatal weight and CRL of the EC-Base group were not different compared to CTRL group. Neonatal weight on PND 10 trended lower in the EC-Nic group compared to CTRL (P = 0.071; (EC-Nic=18.22±0.76 g; EC-Base=19.87±1.16 g; CTRL=21.84±1.09 g). Growth deficits recorded during this study are consistent with an intrauterine growth restriction phenotype most likely attributed vaping nicotine.

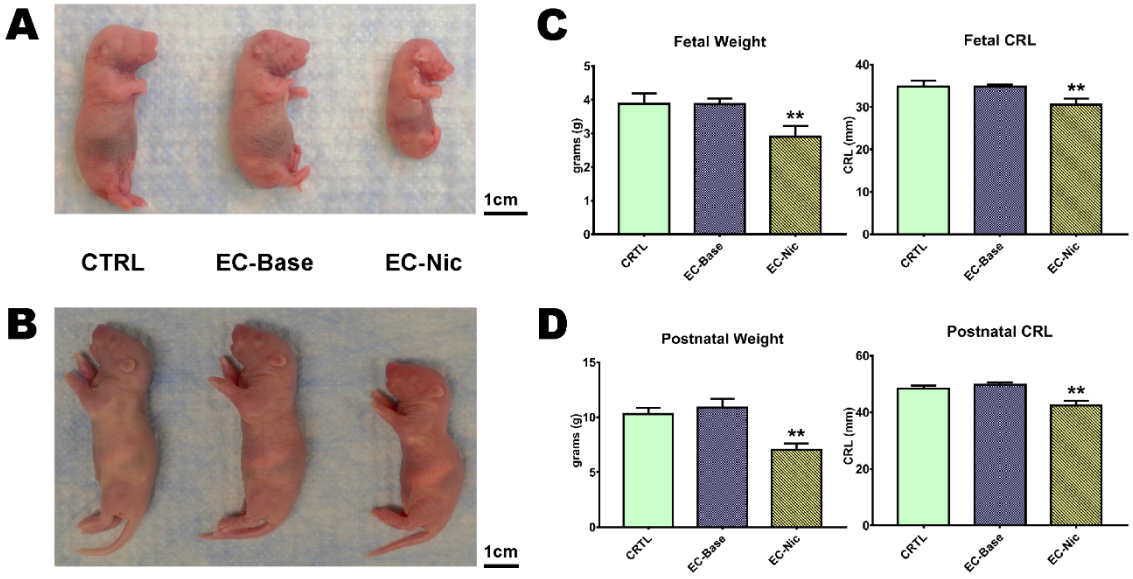

Figure 4-2 Fetal and neonatal growth measures on gestation day 21 and postnatal day 4. (A) Representative images of fetuses following maternal vaping (Order from left to right: CTRL, EC-Base, EC-Nic); (B) fetal weight and crown-rump length; (C) representative images of neonates assessed on postnatal day 4 (Order from left to right: CTRL, EC-Base, EC-Nic); (D) neonatal weight and crown-rump length on postnatal day 4. CTRL = room air control; EC-Base = e-cig aerosol vaping without nicotine; EC-Nic = e-cig aerosol vaping with nicotine. * $P < 0.05$.

4.3.2. Effect of E-Cig Aerosol Vaping on Developmental Lung Transcriptome

To identify molecular targets disrupted by prenatal e-cig aerosol exposure, we examined alterations to the fetal lung transcriptome on GD 21. High throughput RNA-sequencing identified a maximum of 23,483 genes for comparison. The 10 downregulated genes with the greatest $\log_2$(fold change) values in the EC-Nic group compared to CTRL ranged from -3.17 to -4.29 (Figure 4-3A). The 10 upregulated genes with the greatest $\log_2$(fold change) values in the EC-Nic group compared to CTRL ranged from 3.50 to 4.51 (Figure 4-3B). Genes were considered differentially expressed

in the EC-Nic group if the difference in expression compared to the CTRL group was $|\log_2(\text{fold change})| > 1$, and if $P < 0.05$. There were 3,482 genes that were significantly downregulated and 4,466 genes that were significantly upregulated in the EC-Nic group compared to CTRL (Figure 4-3C). A heatmap was constructed to visualize the differentially expressed genes between the EC-Nic group and CTRL group with a $|\log_2(\text{fold change})| > 3$ (Figure 4-3D). A high degree of separation implies maternal vaping of e-cig aerosols containing nicotine can drastically alter the fetal lung transcriptome. Gene ontology (GO) analysis of differentially expressed genes with a $|\log2(\text{fold change})| > 2$ in the EC-Nic group compared to CTRL identified 159 disrupted cellular pathways.

The 10 downregulated genes with the greatest $\log_2(\text{fold change})$ values in the EC-Base group compared to CTRL ranged from -1.11 to -2.28 (Figure 4-3E). The 10 upregulated genes with the greatest $\log_2(\text{fold change})$ values in the EC-Base group compared to CTRL ranged from 1.65 to 1.98 (Figure 4-3F). Genes were considered differentially expressed in the EC-Base group if the difference in expression compared to the CTRL group was $|\log_2(\text{fold change})| > 1$, and if $P < 0.05$. There were 42 genes that were significantly downregulated and 593 genes that were significantly upregulated in the EC-Base group compared to CTRL (Figure 4-3G). A heatmap was constructed to visualize the differentially expressed genes between the EC-Base group and CTRL group with a $|\log_2(\text{fold change})| > 1.5$ (Figure 4-3H). GO analysis of differentially expressed genes with a $|\log_2(\text{fold change})| > 2$ in the EC-Base group compared to CTRL identified 207 disrupted cellular pathways. Significant alterations to the

transcriptome of EC-Nic and EC-Base indicate that chemical constituents other than nicotine in e-cig aerosols may have a negative effect on fetal lung gene expression.

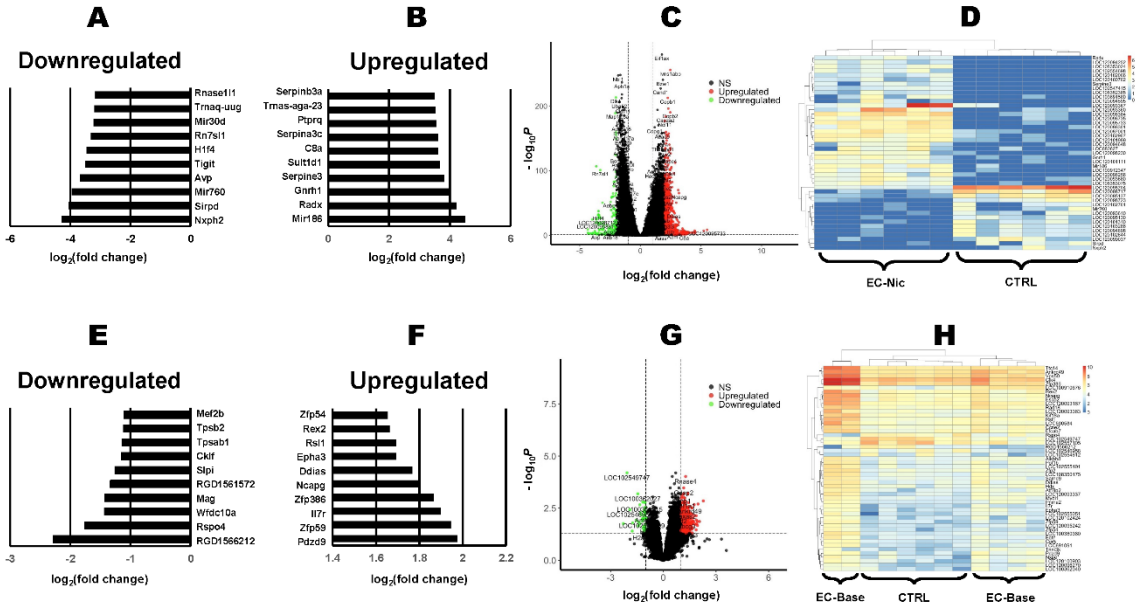

Figure 4-3 E-cig aerosol-induced alterations to whole fetal lung transcriptome. (A) 10 downregulated genes with the greatest log2(fold change) values in the EC-Nic group compared to CTRL; (B) 10 upregulated genes with the greatest log2(fold change) values in the EC-Nic group compared to CTRL; (C) volcano plot of differentially expressed genes in EC-Nic group compared to CTRL with $|\log2(\text{fold change})| > 1$, and $P < 0.05$; (D) heat map of differentially expressed genes in EC-Nic group compared to CTRL with $|\log2(\text{fold change})| > 3$; (E) 10 downregulated genes with the greatest log2(fold change) values in the EC-Base group compared to CTRL; (F) 10 upregulated genes with the greatest log2(fold change) values in the EC-Base group compared to CTRL; (G) volcano plot of differentially expressed genes in EC-Base group compared to CTRL with $|\log2(\text{fold change})| > 1$, and $P < 0.05$; (H) heat map of differentially expressed genes in EC-Base group compared to CTRL with $|\log2(\text{fold change})| > 1.5$. CTRL = room air control; EC-Base = e-cig aerosol vaping without nicotine; EC-Nic = e-cig aerosol vaping with nicotine. * $P < 0.05$.

4.3.3. Effect of E-Cig Aerosol Vaping on Neonatal Pulmonary Morphology

We assessed the weight, volume, and morphology of the neonatal lung to determine the impact of prenatal e-cig aerosol exposure on respiratory development. Representative images of whole lungs and histological sections for each treatment group are shown in Figure 4-4. Fixed lung weight was significantly decreased in the EC-Nic group (0.39±0.07 g) compared to CTRL (0.63±0.03 g; P = 0.004) and EC-Base (0.60±0.03 g; P = 0.01) groups. However, lung weight to body weight ratio was not different across the three groups (Figure 4-4E; EC-Nic=0.05±0.005 g; EC-Base=0.06±0.002 g; CTRL=0.06±0.003 g). Total fixed lung volume in the EC-Nic group (0.45±0.07 mL) was significantly decreased compared to CTRL (0.69±0.03 mL; P = 0.003) and EC-Base (0.71±0.03 mL; P= 0.002) groups (Figure 4-4F).

Alveolar development during early life is a complex and sensitive process that must be tightly regulated to ensure adequate oxygenation of the blood (*175, 187*). To identify changes in the structure of the alveoli, we analyzed sections of the lung by mean linear intercept and radial alveolar count evaluation. Mean linear intercept (MLI) is a commonly used parameter to examine free space within lung parenchyma (*182*). We found that MLI in the EC-Nic (24.03±1.59 μm) group was significantly increased compared to CTRL (15.06±1.66 μm; P = 0.009). The MLI of the EC-Base group (19.06±2.16 μm) was not different from CTRL (Figure 4-4C). Radial alveolar count (RAC) was used to estimate alveolar septation within the lung. We found that RAC in the EC-Nic group (1.69±0.10) was significantly decreased compared to CTRL (2.79±0.25; P = 0.002) and EC-Base (2.376±0.15; P = 0.019) groups, with no difference

between EC-Base and CTRL groups (Figure 4-4D). Morphological changes in the EC-Nic group are consistent with an emphysematic phenotype corresponding to fewer and larger distal air spaces.

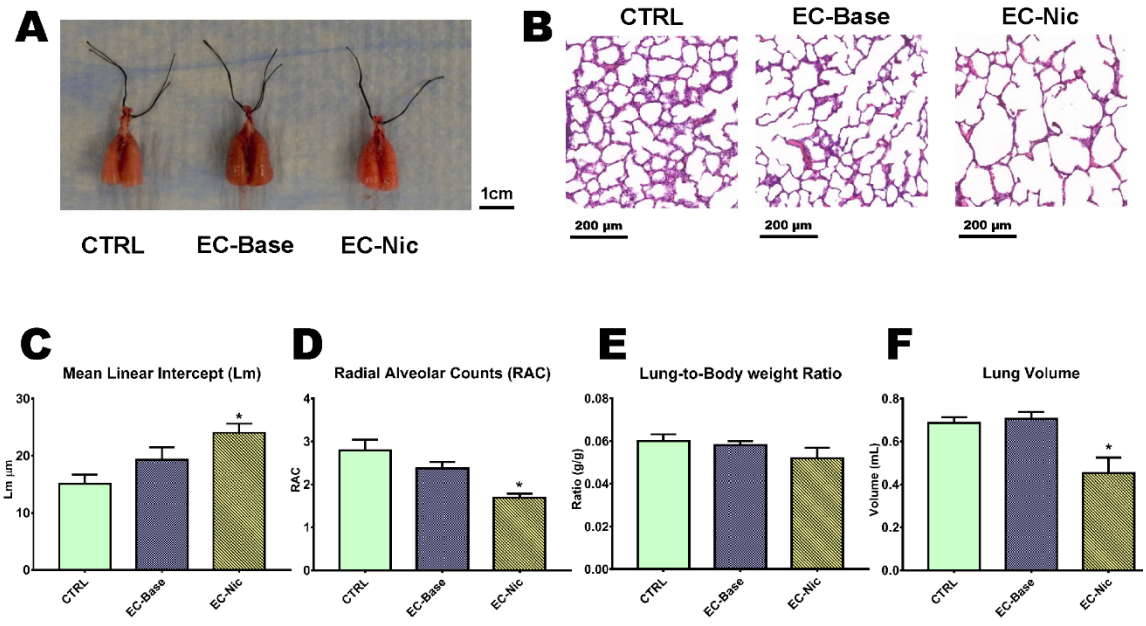

Figure 4-4 E-cig aerosol-induced alterations to morphology of the neonatal lung at neonatal day 4. (A) Representative image of fixative-inflated lungs for histology (Order from left to right: CTRL, EC-Base, EC-Nic); (B) representative images of stained slide sections used for morphological assessment; (C) mean linear intercept; (D) radial alveolar count; (E) lung-to-body weight ratio; (F) fixed lung volume. CTRL = room air control; EC-Base = e-cig aerosol vaping without nicotine; EC-Nic = e-cig aerosol vaping with nicotine. * $P < 0.05$.

4.3.4. Effect of Prenatal E-cig Aerosols on Pulmonary Mechanics

Morphological changes to the lungs are often accompanied by altered respiratory mechanics (*188*). We investigated whole respiratory system and tissue specific pulmonary mechanics on PND 10 to determine functional outcomes of developmental e-cig aerosol exposure. Total respiratory system resistance (Rrs) in the EC-Nic group (0.9025±0.14 cm H2O.s/mL) showed an increasing trend compared to the CTRL group (Figure 4-5A; 0.6262±0.02 cm H2O.s/mL; P = 0.0902). There was no difference in Rrs of the EC-Base group (0.7849±0.04 cm H2O.s/mL) compared to the CTRL group. Total respiratory system elastance (Ers), which represents the resistance to a change in shape of the pulmonary system when pressure is applied, was not different between the three groups (Figure 4-5B; EC-Nic=43.66±10.4 cm H2O/mL; EC-Base=35.76±2.70 cm H2O/mL; CTRL=28.65±1.45 cm H2O/mL). Total dynamic respiratory system compliance (Crs), which represents the ability for the pulmonary system to change shape when varying pressure is applied, was not different between the three groups (Figure 4-5C; EC-Nic=0.02719±0.004 mL/cm H2O; EC-Base=0.02905±0.003 mL/cm H2O; CTRL=0.03556±0.001 mL/cm H2O). However, static compliance (Cst), which represents the ability for the pulmonary system to change shape when a single static pressure is applied, in the EC-Nic group (0.03042±0.004 mL/cm H2O) showed a decreasing trend compared to the CTRL group (Figure 4-5E; 0.04039±0.002 mL/cm H2O; P = 0.0642). There was no difference in Cst of the EC-Base group (0.03343±0.003 mL/cm H2O) compared to the CTRL group. Resistance within the conducting airways, measured as airway resistance (Rn), showed no difference between the three groups

(Figure 4-5D; EC-Nic=0.1056±0.02 cm H2O.s/mL; EC-Base=0.1483±0.01 cm H2O.s/mL; CTRL=0.1353±0.02 cm H2O.s/mL). The area of the pressure volume loop, which estimates the amount of atelectasis prior to the PV loop maneuver, was found to be significantly decreased in both EC-Nic (1.559±0.15 mL.cm H2O) and EC-Base (1.732±0.09 mL.cm H2O) groups compared to the CTRL group (Figure 4-5F; 2.104±0.12 mL.cm H2O; P = 0.006 and P = 0.046 respectively).

Inspiratory capacity, a measure of air volume at a constant pressure (EC-Nic=0.448±0.05 mL; EC-Base=0.471±0.03 mL; CTRL=0.559±0.02 mL), tissue damping, a measure of energy dissipation (EC-Nic=7.465±1.40 cm H2O/mL; EC-Base=6.200±0.35 cm H2O/mL; CTRL=4.898±0.21 cm H2O/mL), and tissue elastance, a measure of energy conservation (EC-Nic=26.783±5.97 cm H2O/mL; EC-Base=22.72±1.75 cm H2O/mL; CTRL=19.09±0.90 cm H2O/mL) were not different across treatment groups. Although several parameters of neonatal respiratory mechanics were not significant, trends in the data suggest that e-cig aerosols with and without nicotine may be deleterious to offspring respiratory health and function.

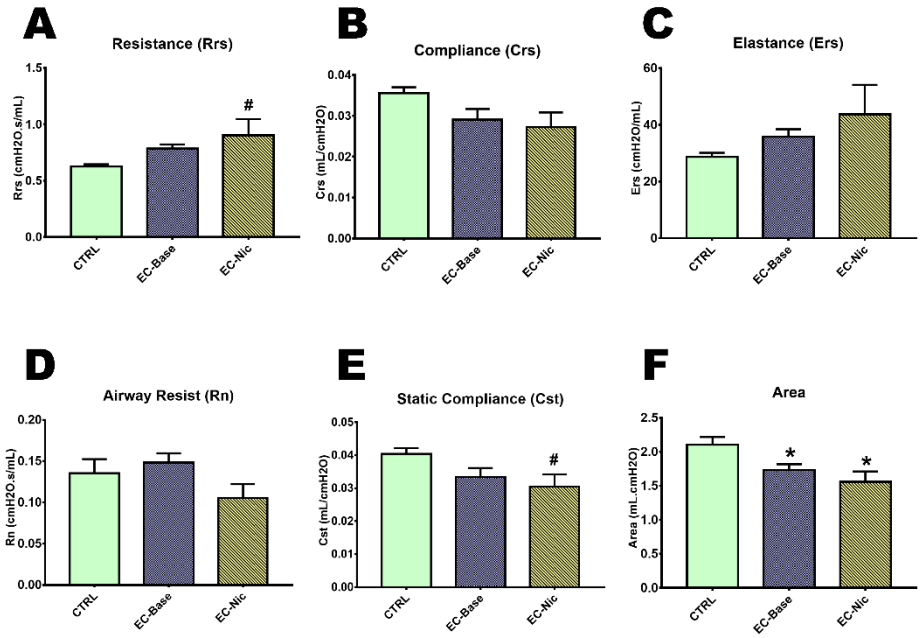

Figure 4-5 E-cig aerosol-induced alterations to neonatal lung mechanics at neonatal day 10. (A) Total respiratory system resistance (Rrs); (B) total respiratory system elastance (Ers); (C) total dynamic respiratory system compliance (Crs); (D) airway resistance (Rn); (E) static compliance (Cst); (F) area of the pressure volume loop. CTRL = room air control; EC-Base = e-cig aerosol vaping without nicotine; EC-Nic = e-cig aerosol vaping with nicotine. * signifies $P < 0.05$. # signifies $0.1 < P > 0.05$.

4.4. Discussion

The developmental impact of prenatal e-cig aerosol exposure on maternal, fetal, and neonatal health is largely unknown. The few animal studies that have been published report e-cig induced dysregulation of the nervous, cardiovascular, and pulmonary systems (*25*). We investigated the effect of prenatal e-cig aerosols with and without nicotine on the developing fetal and neonatal lung. We investigated the effect of prenatal e-cig aerosols with and without nicotine on the developing fetal and neonatal lung through a three-pronged approach to examine the outcomes of exposure at the molecular,

anatomic, and functional levels of the pulmonary system (Figure 4-1). These studies identified significant disruptions to offspring lung development in groups exposed to e-cig aerosols with and without nicotine compared to control. From these novel data we extracted several noteworthy findings: 1) vaping e-cig aerosols significantly dysregulates fetal lung gene expression, which may contribute to malformation of the lungs and development of disease later in life; 2) exposure to prenatal e-cig aerosols with nicotine significantly alters lung structure and restricts alveolar growth and development resulting in an emphysematic phenotype; and 3) respiratory mechanics are significantly altered after developmental vaping of e-cig aerosols in neonatal offspring, which could increase the workload needed for proper respiration. These experiments offer new insight into the developmental effects of gestational e-cig aerosol vaping and evidence that exposure to e-cig aerosols during pregnancy is unsafe.

Lung development begins *in utero* and extends into neonatal life making the lungs a vulnerable target for environmental toxicants over a long period of time (*189*). As the lungs progress through the different stages of development, tight regulation of gene expression and cell signaling are vital to ensure proper growth and organization of this organ (*190*). To assess the impact of prenatal e-cig aerosol exposure on lung development at the molecular level, we examined the transcriptome of fetal lungs via high throughput RNA sequencing. We found that prenatal exposure to e-cig aerosols with and without nicotine significantly dysregulates gene expression in the fetal lungs. Although e-cig aerosols containing nicotine alter the transcriptome to a greater degree than e-cig aerosols without nicotine, it is important to recognize that chemicals other

than nicotine have a significant effect on developmental processes at the cellular level. When we examined the top 10 downregulated and upregulated genes of both groups exposed to prenatal e-cig aerosols we noted that several of the dysregulated genes play regulatory roles in the development of lung cancers, chronic obstructive pulmonary disease (COPD), and asthma. Of the 10 upregulated genes with the highest fold-change values in the EC-Nic group, three genes encode serine protease inhibitors (serpins), *Serpinb3a*, *Serpina3c*, and *Serpine3*, which are a class of molecule that irreversibly inhibit proteolytic activity (*191*). The products of these genes are important for tissue homeostasis within the lung, however, overexpression has been associated with increased inflammation and may be a contributing factor to the development of COPD and lung cancers (*192-194*). In the EC-Base group, two of the top 10 downregulated genes encode for tryptases, which are the most abundant product of the mast cells and contribute to the development of asthma and fibroblast proliferation in the lung (*195-197*). R-spondin 4 (*Rspo4*) was also among the top 10 downregulated genes in the EC-Base group. *Rspo* has been shown to have a potentiating effect on the *Wnt*/β-catenin pathway, which regulates cell proliferation, differentiation, and maintenance (*198, 199*). Interestingly, a recent study on the effect of prenatal e-cig aerosols with flavorings and nicotine in mice reported downregulation of 75 genes in the *Wnt* signaling pathway and related them to decreased growth and proliferation (*123*). The data collected in the current study further corroborate recent findings on the effects of gestational e-cig aerosol exposure and provide support for a potential mechanism of altered pulmonary development.

Alveolar development is a complex process that begins at approximately 36 weeks in utero in humans (*200*). The equivalent developmental stage of the neonatal rat lung occurs at neonatal day 4 (*201*). This time point is marked by the formation of secondary septa, which were utilized in this study to assess the impact of prenatal e-cig aerosols on alveolar growth. Although weight on PND 4 was significantly decreased in the EC-Nic group compared to CTRL, the lung-to-body weight ratio was not different. Therefore, exposure to prenatal e-cig aerosols did not inhibit lung growth to a greater degree than the reduction in whole body growth. Fixed lung volume in the EC-Nic group was significantly decreased compared to CTRL and most likely attributed to the overall reduction in size induced by prenatal e-cig aerosols. Despite reductions in the size of the lungs, there was a significant increase in the MLI of the EC-Nic group compared to CTRL, indicating an increase in the size of distal air spaces. This finding accompanied by reduction in RAC of the EC-Nic group compared to CTRL demonstrate characteristics of an emphysematic phenotype with larger and fewer distal air spaces. Prenatal exposure to e-cig aerosols in mice was previously shown to increase MLI and reduce cellular proliferation at PND 10 (*18*). In adult mice that were prenatally exposed to e-cig aerosols containing nicotine and flavorings, there was a similar increase in MLI of the lung that was associated with altered epithelial cell differentiation (*123*). Our data is the first to demonstrate that vaping e-cig aerosols containing nicotine, without the confounding factor of flavorings, can have a detrimental effect on the structure of the neonatal lung. Combined, these data raise concern for the development of pulmonary-

related diseases in later life of individuals who have been prenatally exposed to e-cig aerosols.

Exposure to tobacco products and nicotine during gestation is known to increase the risk of sudden infant death syndrome, respiratory distress, and asthma in humans (*1*). A longitudinal study investigating the effects of prenatal tobacco product exposure on offspring respiratory function after 21 years of life found a strong correlation between exposure and reduced expiratory flow rate (*202*). It was previously observed that a loss of respiratory function at an early age may persist into later life (*203*). Respiratory function is inherently connected to the mechanical properties of the pulmonary system as a whole. This is the first study to examine the effect of prenatal e-cig aerosol exposure on neonatal pulmonary mechanics. We found a significant decrease in the area of the pressure-volume curve of offspring exposed to prenatal e-cig aerosols with and without nicotine, which provides an approximation of the amount of atelectasis prior to the ventilation maneuver. We also report an increasing trend in respiratory system resistance and a decreasing trend in static compliance in the EC-Nic group compared to CTRL. An increase in system resistance and a decrease in compliance lead to an increase in labor of breathing and may contribute to neonatal hypoxia or respiratory distress. In a recent mouse study, the elastance of the respiratory system in pregnant mother exposed to e-cig aerosols was significantly increased compared to room-air controls (*123*). There have been several human and animal studies demonstrating the effects of e-cig aerosols on adult respiratory function, yet there are no current evaluations in neonates or offspring exposed to e-cig aerosols during gestation.

The studies presented herein display strong evidence to support that exposure to prenatal e-cig aerosols with and without nicotine can have a deleterious effect on fetal and neonatal pulmonary development and respiratory health. Utilizing a three-pronged approach, we were able to identify e-cig aerosol-induced alterations to the developing lung at the molecular, anatomic, and functional levels. This was the first study to examine genome-wide alterations to the transcriptome of the fetal lung and the first study to assess neonatal pulmonary mechanics in offspring exposed to gestational e-cig aerosol vaping with and without nicotine. The alterations reported in the current investigation indicate that human consumption of e-cigs during pregnancy may have detrimental effects on infant respiratory health that may persist through adult life. This study also exemplifies the need for the implementation of public health policy and regulation of e-cigs in the context of pregnancy. Further research is required to delineate molecular mechanisms of e-cig aerosol-induced pulmonary deficits and to identify therapeutic targets to assist individuals exposed to e-cig aerosols during pregnancy.

5. CONCLUSIONS

5.1. Effect of E-Cig Aerosols Exposure on Pregnancy and Development

We have demonstrated herein that chronic exposure to e-cig aerosols during pregnancy can have deleterious effects on both the mother and the fetus. Mass spectrometric analysis of the e-cig aerosols generated in this study detected a larger number of chemical constituents than were originally present in the e-cig liquid used (propylene glycol, glycerol, and nicotine). The additional chemicals produced by the vaporizing process included carcinogens, such as polycyclic hydrocarbons, which may pose an immediate risk to e-cig users. The level of nicotine utilized in these studies produced a median serum nicotine concentration that was roughly equivalent to daily moderate/heavy human smokers (*34, 76, 135, 136*). This allowed for the evaluation of the effects of nicotine at a physiologically relevant dosage.

In all studies, offspring of animals exposed to e-cig aerosols with nicotine displayed an intrauterine growth restriction phenotype during late gestation and early life. Reduced fetal and neonatal growth is a strong indicator of developmental deficiencies and is correlated to an increased risk for adult-onset diseases (*70, 186*). The data suggest that nicotine is the main constituent driving the reduction in growth since there was no difference in the size of offspring exposed to prenatal e-cig aerosols without nicotine compared to controls. Blood flow in the material uterine artery is positively correlated to fetal growth (*70, 71, 75*). Animals exposed to e-cig aerosols with nicotine were found to have reduced blood flow in the maternal uterine artery and fetal

umbilical artery. Although not significant, animals exposed to e-cig aerosols without nicotine showed a decreasing trend in blood flow through these vessels, demonstrating an effect due to chemicals other than nicotine. Nicotine acts as a potent vasoconstrictor which may inhibit normal maternal uterine artery adaptations during pregnancy, and lead to intrauterine growth restriction.

Proper growth and development during pregnancy relies on the constant flow of nutrients from the mother to the fetus. Exposure to tobacco products during pregnancy has been shown to reduce the transfer of vital compounds such as amino acids across the placenta (*154-156*). Amino acids are crucial to fetal protein synthesis, cell signaling, and homeostasis (*124, 125*). We found that animals exposed to e-cig aerosols with nicotine have altered concentrations of amino acids in the maternal and fetal plasma. All amino acid concentrations that were different from the controls' were significantly increased. In humans, an increase in maternal plasma amino acid concentrations has been correlated to reduced offspring weight at birth (*129, 150*). Nicotine may inhibit the flow of amino acids across the placenta resulting in an accumulation of amino acids in the maternal plasma. Exposure to e-cig aerosols with and without nicotine disrupted the amino acid profiles in both male and female fetal lungs. The fetal lungs are a sensitive target organ for prenatal tobacco product exposure (*157*). We noted an increase in the concentrations of amino acids associated with the urea cycle (citrulline, aspartate, arginine, and ornithine) in the fetal lungs of offspring exposed to prenatal e-cig aerosols with nicotine. Arginine is also necessary for nitric oxide synthesis which is an important signaling molecule in fetal lung morphogenesis (*164-166*). If arginine is redirected from the nitric

oxide pathway to the urea cycle we may anticipate prenatal e-cig aerosol-induced respiratory pathologies in neonatal and adult life.

We continued to investigate the effects of prenatal e-cig aerosol exposure on offspring lung development by implementing a multi-level approach. At the cellular level we assessed gene expression in whole fetal lungs via high throughput RNA sequencing and found that e-cig aerosols with and without nicotine alter fetal lung transcriptome. Differentially expressed genes were found to be associated with inflammation and development of asthma and chronic obstructive pulmonary diseases. Downregulation of genes associated with the *Wnt* signaling pathway, necessary for normal organogenesis of the lungs, were also reported in offspring exposed to e-cig aerosols with and without nicotine (*204*). At the anatomical level we examined the morphology of the lung, focusing on the structure of the alveoli. We found that exposure to e-cig aerosols with nicotine resulted in fewer and larger distal air-spaces consistent with an emphysematic phenotype. Reduced alveolar proliferation may inhibit normal lung function and predispose the offspring to the development of COPD later in life. To determine the functional effects of prenatal e-cig aerosol exposure we utilized several forced oscillation techniques to measure parameters of neonatal pulmonary mechanics. We found that the only parameter significantly altered in animals exposed to e-cig aerosols with and without nicotine was the area of the pressure volume loop, which is an approximation of the amount of atelectasis prior to the inflation maneuver. We also saw a trend of increased respiratory system resistance and decreased static compliance in offspring exposed to prenatal e-cig aerosols with nicotine. Alterations in these

parameters may increase the labor of breathing in these offspring and lead to respiratory complications. Taken together, the reported findings from each of these studies support the notion that exposure to prenatal e-cig aerosols can have potentially harmful outcomes for both the mother and the fetus.

5.2. Future Directions

5.2.1. Mechanisms of E-Cig Induced Sequelae

These studies have laid the foundation for our understanding of how e-cig aerosols impact pregnancy and development, however, the precise mechanisms of these physiological alterations are unknown. Future studies will be required to delineate the mechanism of e-cig aerosols-induced growth restriction. Nicotine, on its own, is known to inhibit fetal growth but it is unclear whether this results from reduced blood flow to the utero-placental compartment or the disruption of cellular pathways by nicotine in the fetus. The flow of nutrients from the mother to the fetus by the placenta following e-cig exposure will also need to be assessed to determine how e-cig aerosols influence amino acid transporters.

Pulmonary development is a complex process that begins in utero and ends during postnatal life (*189*). The pulmonary alterations described in these studies establish that chemical constituents other than nicotine can negatively impact offspring respiratory development. Respiratory deficits attributed to prenatal tobacco product exposure are usually life-long and increase the risk of disease in adulthood (*1, 14*). Further analysis of RNA-seq data is needed to identify canonical pathways effected by e-cig aerosol exposure. There are a number of factors that play a role in normal lung

development, including inflammation, extracellular matrix remodeling, and vascularization (*175*). Once specific pathways targeted by e-cig aerosols have been identified we may attempt to recapitulate e-cig aerosol-induced pulmonary sequelae via the activation or inhibition of these pathways. These studies will be necessary to develop strategic therapeutic interventions for individuals exposed to prenatal e-cig aerosols.

5.2.2. Animal Studies

Current animal models of prenatal e-cig aerosol exposure include rodents, amphibians, and fish (*25*). These models offer insight into some of the effects of e-cigs on pregnancy and development, however, the developmental time-line of the organ systems in these animal models do not exactly match that of humans and may confound results depending on which aspect of development is being assessed. Alternate animal models may be necessary to replicate a more accurate physiological response to prenatal e-cig aerosols. Current reports of e-cig aerosol-induced effects on development identify significant alterations to fetal pulmonary, cardiovascular, and nervous systems. More work will need to be done to examine the effect of e-cigs on other organ systems, such as the immune, hormonal, and digestive systems. Whereas many current studies evaluate offspring, it will also be important to perform longitudinal studies to examine how developmental deficiencies at birth impact later life.

Regardless of which animal model is used, the effect of prenatal e-cig aerosols on maternal health is often overlooked. In order for e-cig aerosols to have an effect on fetal development they must first be inhaled by the mother through first- or second-hand exposure. Normal human pregnancy is accompanied by a large number of physiological

adaptations that are necessary for proper development of the fetus (*205*). The direct exposure to e-cig aerosols is of great concern for this vulnerable population, and future studies will need to incorporate the maternal physiological response in their interpretations.

In addition to investigating the maternal response to prenatal e-cig aerosols, the confounding factor of e-cig liquid flavorings and composition will need to be considered. We removed flavorings from our studies to focus our research on the main chemical constituents in most commercially available e-cig liquids, however, nearly all commercially available e-cig liquids contain a small percentage of flavorings. Flavorings are incredibly diverse in their chemical composition and can greatly increase the number of new chemicals that are generated during the vaporization of the e-cig liquid. The proportions of propylene glycol and glycerol also plays a role in the composition of the aerosol produced and can vary between commercially available e-cig liquids. In order to standardize the study of prenatal e-cig aerosol exposure, it will be necessary to characterize how flavorings and composition effect the aerosol produced and to identify a single standard e-cig liquid to be used in research.

5.2.3. Human Studies

To our knowledge, there is currently only one human study investigating the effects of e-cigs and pregnancy. The study reports that exposure to e-cig aerosols during gestation increases the risk of low weight at birth (*174*). The novelty of e-cig vaping has created a sizeable knowledge gap on the effects these products have on human physiology. Although there is some data exploring the effects of e-cig vaping on adults

there is almost no data for vulnerable populations such as pregnant women. The perception that e-cig vaping is a safer alternative to traditional cigarette smoking or that e-cigs can be effectively used as a tobacco product cessation tools may lead to an increase in the use of e-cigs during pregnancy. The exact number of pregnancies that have been exposed to e-cig aerosols is unknown, but is thought to be approximately 4% in the United States (*206, 207*). More work will need to be done to accurately determine the severity of the gestational e-cig exposure. Additionally, large longitudinal cohort studies should be initiated now to identify individuals who have been exposed to prenatal e-cig aerosols. Maternal and offspring biometrics should be included in these studies since maternal biological factors may play a role in the effect of e-cig exposure. Longitudinal studies, similar to the Melbourne asthma cohort, will be critical to understanding how exposure to e-cigs in early life impact health over the course of a lifetime (*203*). These studies will serve as a framework for detecting health complications attributed to prenatal e-cig aerosols in humans and may improve or expedite the development of therapeutics needed to prevent future health concerns.

5.2.4. Combined Exposure

Recent reports reveal that a majority (70%) of women who use e-cigs during pregnancy also consumed traditional tobacco cigarettes (*206, 208*). The combined use of these two tobacco products is most likely due to the perception that e-cigs are safer than cigarettes and that there is less social stigma associated with e-cigs than there is with traditional cigarettes. Exposure to nicotine in utero by e-cigs and traditional cigarettes may exacerbate deficiencies that have been previously reported. Therefore, in addition to

the study of e-cig exposure alone, future studies will need to investigate the effects of combined use of e-cigs and traditional cigarettes to decipher which health outcomes are associated with each tobacco product.

The use of any tobacco products during pregnancy increases the risk of dual substance use, and is typically accompanied by consumption of alcohol (*209*). While there are many reports on the effects of either alcohol or tobacco use during pregnancy there are no studies that investigate the comorbidity of alcohol and e-cig consumption during pregnancy. We previously examined the effects of dual exposure to prenatal e-cig aerosols and alcohol on offspring growth and pregnancy related hemodynamics in a rodent model. We found that dual exposure to e-cig aerosols and alcohol during pregnancy produced similar growth restriction as both e-cig only exposure and alcohol only exposure, however, placental efficiency, maternal uterine artery blood flow, and fetal umbilical artery blood flow followed trends that were more similar to alcohol exposure alone (Figure 5-1). These preliminary findings should be considered in the design of future experiments and exemplify the need for more research on the dual exposure of alcohol and e-cig aerosol exposure during pregnancy.

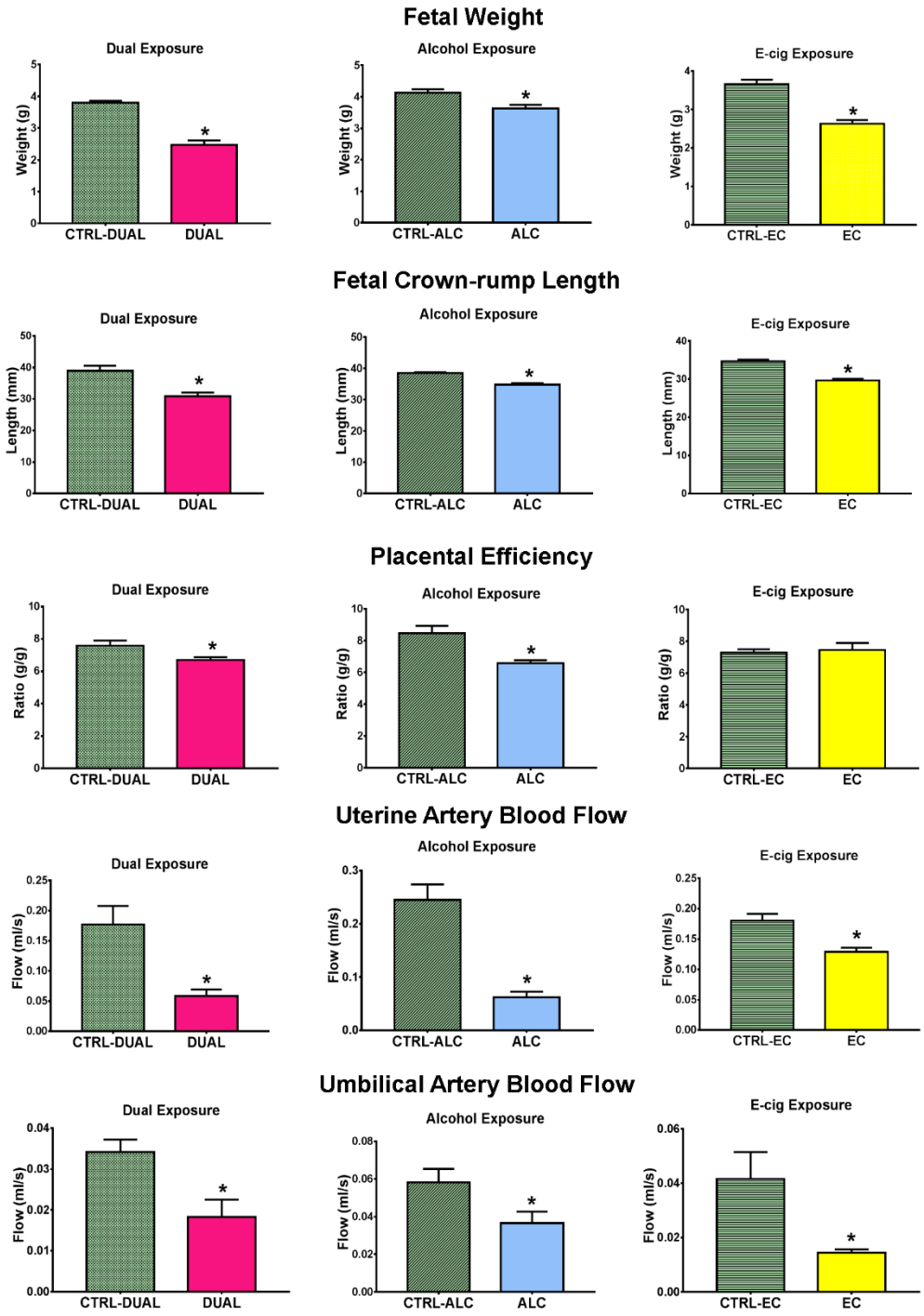

Figure 5-1 Effects of chronic dual exposure to prenatal e-cig aerosols and alcohol on offspring growth and pregnancy related hemodynamics. CTRL = control; EC = prenatal e-cig aerosol exposed; ALC = prenatal alcohol exposed; DUAL = dual prenatal e-cig aerosol and alcohol exposed. * P<0.05 compared to exposure-specific control.

5.3. Health Policy

The United States Surgeon General released a statement in 2016 stating that e-cig use, especially among vulnerable populations such as teens and young adults, has skyrocketed (*2*). In the past, advertisements for the sale of e-cigs were directed at teens and young adults by including images of younger people using these products. Many producers of e-cigs used enticing flavors in their e-cig liquids and profited from unsubstantiated claims that e-cigs are less harmful than cigarettes. The implementation of legislation has since banned targeted advertising of e-cigs to young adults, restricted access to e-cig products by people under the age of 18, and has also regulated the sale of specific flavors of cartridge based e-cigs. These regulations have helped to better inform the public of e-cig related safety, yet the use of e-cigs among vulnerable populations remains an issue at large.

5.3.1. Product Regulation

E-cig liquids contain varying amounts of nicotine that range from 0 mg/mL to over 50 mg/mL in the United States (*210*). Higher concentrations of nicotine in these liquids has been shown to increase the delivery of nicotine to the user and may cause addictive use behavior (*76, 80, 81*). At these levels of nicotine, e-cigs have the potential to delivery more nicotine that traditional cigarettes (*3, 4*). There are currently no nicotine concentration restrictions in the United States, but European law has capped the level of nicotine at 20 mg/mL (*211*). A review of these regulations in the United States is scheduled for the year 2022. To address the health consequences related to nicotine consumption and to curb nicotine-dependence of current e-cig users the federal

government should enact legislation that caps nicotine concentration in commercial e-cig liquids.

In 2021, the National Youth Tobacco Survey found that 85% of current youth e-cig users were using flavored e-cig liquids (*212*). In an effort to reduce the use of e-cigs by teens and young adults, the federal government of the United States banned the sale of non-tobacco flavored e-cig cartridges. However, flavored e-cig liquids are still available for purchase as refill liquids in many commercial vape shops. The variability of e-cig liquid flavorings is continually growing and access to these products by young adults is still a serious concern. Tighter regulation on the quality and type of flavorings that are available is needed to minimize the use of e-cigs by youth and to mitigate the potential health impact e-cigs pose to society.

5.3.2. Physician Guidelines

There is a large focus on combatting the use of e-cigs by youth and young adults. The current recommendations offered by the CDC strongly discourage non-smokers from using e-cig/vaping products (*213*). The CDC asserts that adults who use traditional tobacco cigarettes should consult their primary care physician before switching to e-cigs. While physicians are encouraged to continue reporting e-cig/vaping associated lung injury in patients, the guidelines for e-cig use as a cessation tool or during pregnancy are unclear. Due to the misconceptions surrounding e-cigs, some users may not consider e-cig vaping devices as tobacco products and may unintentionally fail to disclose the use of e-cigs to their physicians. Hospitals should require physicians to ask patients specifically about e-cig use and exposure to avoid confusion about e-cigs' status as a

tobacco product. Based on findings in human and animal models of e-cig aerosol exposure, physicians should not recommend the use of e-cigs or any other tobacco product during pregnancy. Recording and reporting of e-cig use during pregnancy by physicians should be made standard practice to improve care strategies for both the mother and the developing fetus.

5.4. Final Remarks

Despite known health consequences of tobacco products, the use of e-cigs by young adults and the perception that e-cigs are less harmful than traditional cigarettes has led to the use of e-cigs during pregnancy. Through extensive investigation we have demonstrated that exposure to prenatal e-cig aerosols can have a damaging effect on maternal and fetal physiology. It is important to recognize that chemical constituents other than nicotine in e-cig aerosols may contribute to e-cig aerosol-induced alterations to pregnancy. Our data also corroborate the claim that the developing fetal lungs are a sensitive target organ of prenatal tobacco product exposure. Future studies are needed to determine an exact molecular mechanism of the prenatal e-cig aerosol-induced changes reported herein, and should take into consideration any confounding factors such as flavorings, e-cig liquid composition, and nicotine concentration. The evidence presented in these studies will help to improve clinical recommendations given by physicians and legislation governing the regulation of e-cigs/vaping products.

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