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# Histological evaluation of offspring kidneys following prenatal vaping exposure.

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By

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## INTRODUCTION

Cigarettes have been extensively researched starting in the late 1920s with the first report from the Surgeon General published in 1964 (United States Public Health Service Office of the Surgeon General, 1964). Since then, smoking has been proven to be detrimental all around, as it contains seventy identified carcinogens such as nitrosamines and heavy metals (Hecht & Hatsukami, 2022). This has caused various nicotine-containing products to be developed to replace cigarettes, such as electronic cigarettes, pipes, and smokeless tobacco, with the first being the more common one. These alternatives enter in either the respiratory category, such as pipe and electronic cigarette, or the oral category, such as smokeless tobacco, with both having a different route of entry in the body, and thus may affect various organs differently.

Starting in the 1960s, the US Surgeon General Reports has published important government reports regarding the figures of cigarettes usage, going over the available research and summarizing the current knowledge on various topics relating to smoking, such as health consequences on various groups, methods of prevention, tobacco use in various groups, and more recently electronic cigarette usage and smoking cessation (U.S. Department of Health & Human Services, 2022). They indicate that the prevalence of smoking among adults in the United States has decreased from 68% in 1955 to 28.4% in 1990 to 15.8% in 2017 for men and from 32.4% in 1955 to 22.8% in 1990 to 12.2% in 2017 for women (Centers for Disease Control, 1992; United States Public Health Service Office of the Surgeon General, 1964; United States Public Health Service Office of the Surgeon General, 2020). 1990 is an important year for tobacco use, as it is the year smoking was banned from public transportations, and that the Surgeon General reported on smoking cessation having “major and immediate health benefits for men and women of all ages” (Centers for Disease Control, 1992). Despite this decrease in

relative number of smokers in the United States, there are still new smokers in the global population as there was 0.99 billion smokers in 1990 and 1.14 billion smokers in 2019 worldwide (Reitsma et al., 2021). This number has increased despite the development of those alternatives, some of which are increasingly used by adolescents in the United States, especially high school students. Among those students, 16% are currently or have used electronic cigarettes, with 25.3% of high school students using any form of tobacco products (Makadia, Roper, Andrews, & Tingen, 2017). Furthermore, it is still unclear whether some of the new alternatives, especially e-cigarettes, are helping when quitting cigarettes or not, and whether their use by adolescents represents any risk for cigarette usage later in life (United States Public Health Service Office of the Surgeon General, 2020). Because an increase in tobacco usage this early is known to be more correlated with tobacco usage later in life, this increase in e-cigarette usage in adolescents could then theoretically lead to a greater number of persons either continuing to vape or switching to cigarettes later in life (Makadia et al., 2017).

The electronic cigarette is now being used along with cigarettes, either replacing it or being used as a supplement. This device's refill liquid contains nicotine, the same addictive chemical as in cigarettes, fewer combustion products such as Carbon Monoxide, and less toxic chemicals such as carcinogens, including benzene, arsenic, acetaldehyde, and chromium among others (Hecht & Hatsukami, 2022). Those devices are also advertised as a "safe" smoking cessation aid in both the United States and in the United Kingdom. As a result of this advertisement, 71% of pregnant women who switched from smoking to vaping did so on the belief that it would help them quitting (DeVito et al., 2021a). However, those pods are not limited in their concentration of nicotine in the United States, which is not the case in the United Kingdom where they have been limiting it (CNBC, 2019). Finally, according to one of the major

conclusions of the Smoking Cessation Report from the Surgeon General in 2020: “there is presently inadequate evidence to conclude that e-cigarettes, in general, increase smoking cessation”(United States Public Health Service Office of the Surgeon General, 2020).

As for the safety of the electronic cigarette, studies have proven reduction in alterations compared to cigarettes in kidneys, but no study has determined that electronic cigarettes were safe for use during pregnancy (Li et al., 2019). Furthermore, some carcinogens have been identified in vape pods, some of which are common with cigarettes, such as acetaldehyde, acetone and other breakdown products of glycerol and propylene glycol, both present in e-cig refill liquid, and some heavy metals such as lead and iron (Gordon et al., 2022). As some pregnant women report switching from cigarettes to e-cigarettes on the belief that it would cause less harm on the developing child, if any harm, studies have been performed to verify this assumption. However, few studies exist showing that electronic nicotine delivery systems (ENDS) are less toxic compared to smoking. Most experiments available today highlight similar altered birth outcomes to cigarettes, such as small for gestational age children among others (DeVito et al., 2021b).

The physiological role of the kidney is to filter blood and remove harmful components. The kidney is made up of individual filtering units called nephrons. Each nephron is made up of a glomerulus, where the blood goes through the capillaries, surrounded by a Bowman capsule, whose purpose is to absorb compounds smaller than proteins pushed out by the blood through the hydrostatic pressure. These glomeruli are only observed in the outer portion of the kidney: the cortex. The medulla, or the inner portion, contains the loops of Henle along with tubules and collecting ducts. These compounds are then taken into the proximal tubule, where some are reabsorbed into the blood, such as glucose. The rest is taken through the loop of Henle, which

reabsorbs salt and is responsible for the maintenance of the salt gradient of the kidney. Then, in the distal tubule, some compounds are reabsorbed, such as any remaining salt being reabsorbed under hormonal control. The remaining urine is then taken into the collecting duct, where some of the water is reabsorbed depending on the presence of water channels on the cells and the salt gradient, and the more concentrated urine is transported to the bladder where it is excreted (Wallace, 1998).

Our current study focuses on the impact of gestational electronic cigarette inhalation exposure on the development of the kidney in rodents. The first thousand days of development of an embryo, including both prenatal and neonatal development, has been found to be critical to the future outcome of the body, especially in kidneys, as certain stress may result in either permanent damage early on in development, or may lead to some irreversible epigenetics changes and lead to defects which will result in further damage (Hsu & Tain, 2021). These changes include a low nephron number, altered glomerulus size, and some oxidative damage. If less glomeruli are present, then less blood is capable of being filtered by the kidney in a given amount of time, and this may cause an increase in blood pressure, resulting in further damage, whether it be oxidative or not, and resulting in the condition known as chronic kidney diseases (CKD) (Puelles et al., 2011).

This series of events directly applies the fetal origins hypothesis which states that early changes in the fetal in-utero environment, such as undernutrition, may lead to less nutrients being attributed to side organs, as the main organs such as the heart and the brain are the primary focus. This hypothesis is also known as the Barker's hypothesis, named after his main contributor David Barker. These alterations in turn result in abnormal or lesser development in those side organs, including the kidney, and may result in chronic diseases later in life. This may also

happen even if the offspring is born with a larger weight at birth, if a toxin was present during development (Kimm, 2004). Following the works of David Barker, Barry Brenner developed a hypothesis, named after him, which states that factors impacting the number and size of glomeruli, such as aging or developmental issues, will result in hypertension through a vicious cycle (Didion, 2017). As a result, it implements Barker's hypothesis and describes the mechanism given above, with the lack of glomeruli and the development of CKD as a result of this oxidative damage and those epigenetic changes early on in development. Given that the children of smoking mothers are born with a reduced weight, this indicates that there may be a limiting amount of nutrients in the placenta which will be given to certain prioritized organ systems. Those children also end up more often with diseases such as CKD and hypertension, validating these hypotheses (Jebasingh & Thomas, 2022). This stressor may also induce kidney genetic reprogramming of biochemical pathways, such as gluconeogenesis, due to certain cells not being present in required amounts, resulting in an imbalance in those pathways. This series of events can turn into more chronic diseases in the long term. This is important to note that along with its filtrating purpose, the kidney is the only organ aside from the liver being capable of undergoing gluconeogenesis, which is the production of glucose molecules when the blood sugar gets low. Thus, if any damage occurs, this may impact the normal functioning of the blood glucose regulation system (Tain & Hsu, 2017).

Much is known about the developmental toxicity of cigarette smoke. For example, it is known that prenatal maternal smoking causes low birth weight in the offspring in both humans and mice (Rogers, 2019). This low birth weight induces further developmental issues, supporting the Barker's and Brenner's hypotheses. In a rodent model, prenatal inhalation exposure to cigarettes has been shown to be detrimental in the development of the kidney in early life,

resulting in functional deterioration in adulthood. In this study, animals were exposed twice daily to the smoke produced by two cigarettes, for 5 minutes. A key result was in average glomerular size, as smoke exposed animals had a larger glomerulus on average at postnatal day 20 (PD20), but a smaller glomerulus on average at postnatal week 13 (W13). Moreover, in all animals at all ages tested, the glomerulus number was lower in the offspring exposed to cigarette smoke prenatally. Finally, renal growth factors and transcription factors have been found to be misregulated in animals having been exposed to cigarette smoke. Different inflammation and injury markers were also found in higher quantities in the smoke exposed offspring, which could explain the damage. Thus, smoking does impact the renal development of the offspring, and predisposes the offspring to chronic kidney disorders (Al-Odat et al., 2014). These results also strengthen the idea that early exposure to toxic compounds could result in chronic kidney disease, as the proper development of the renal tissue prevents these diseases, and any alteration could result in subsequent disorders later in life (Hsu & Tain, 2021; Tain & Hsu, 2017).

As for electronic cigarettes themselves, a few studies point to their potential impact on the kidney development in rodents. In mice, an oral exposure to nicotine for 4 weeks, present in the water for this study, seemed to affect the kidney development differently based on the timing of the exposure and its concentration. The earlier in life the exposure occurred, the more damage the kidney received, which could potentially lead to some chronic kidney diseases later (Arany, Hall, & Dixit, 2017). However, this specific study did not involve any prenatal exposure as the animals, which were C57Bl/6J mice, were 4 weeks and 24 weeks old, nor was it an inhalation exposure. In rats, nicotine has been shown to have an even greater impact during fetal development. The nicotine was intraperitoneally injected in the pregnant dams with a given daily dose of 2.1 mg via osmotic pumps, and two strains of rats were tested, the SHR and the BN. In

this experiment, it was found that 9 weeks after birth, nicotine exposure during fetal development in SHR rats led to a moderate decrease in kidney size and in total glomerular mass, and also caused an increase in *Agtr1b* expression, which may result in hypertension later in life (Toledo-Rodriguez et al., 2012).

The refill liquid used in electronic cigarettes was tested on Wistar rat adults and was found to have detrimental effects on their kidneys when introduced intraperitoneally for 4 weeks, regardless of the presence of nicotine (Golli et al., 2016). The impact of electronic cigarettes compared to cigarette smoke on renal development in mice through a prenatal inhalation exposure was assessed using Balb/c mice and either exposed the dams to cigarette smoke, to e-cigarette vapors, to both with a switch after mating, or to filtered air. Animals were exposed 6 weeks prior to mating to their assigned group until weaning, and the kidneys were analyzed 1 day, 20 days, and 13 weeks after birth. The main result of the experiment was that vaping was less detrimental to kidneys development than cigarettes alone. There were still defects observed in the kidneys compared to shams, such as increased oxidative stress, inflammation, and fibrosis factors, and in e-cigarette replacement group and cigarette exposed pups, a lower density of glomeruli, which may result in hypertension later in life. However, no significant histological alterations were found. It is important to note that the mice were exposed to smoke generated by two cigarettes twice daily in the smoke exposed group, and to the equivalent in e-cigarette vapor with pods containing 18 mg/mL of nicotine. Thus, although this setup imitated one of a light smoker, there was already some observed effects on the density of glomeruli (Li et al., 2019).

Despite these findings and the lack of further testing, many pregnant women still decide to switch to electronic cigarettes. The extent to which ENDS induce damage in the offspring is still unclear in humans, and the current evidence points to the presence of oxidative damage and

altered development in mice kidneys, as with prenatal cigarettes exposure, due to similar compounds being present. There are also reports that even prenatal exposure to nicotine-free electronic cigarettes might also impact the development of the offspring, and thus could have additional toxic compounds in need to be identified and investigated (Greene & Pisano, 2019). Studies in humans have validated the results found in rodent models, as those are consistent with the findings in children having been exposed to cigarettes prenatally, with similar outcomes such as low birth weight, impaired kidney development with lower glomeruli density, among other factors (Kooijman et al., 2015). Thus, the rodent model to study prenatal exposure has been proven to be reliable and relatable to humans, despite some key anatomical and developmental differences such as lobe numbers in the kidney, the timing of development, among others, which are important to keep in mind when analyzing the results (Lindström et al., 2018).

**METHODS AND PROCEDURES**

All animal handling and associated procedures were approved by IACUC at the University of Louisville. Before starting exposure, the females were separated into two groups: the vaping exposed group (Vape), and the Sham group, or the group exposed to filtered air. Adult C57BL/6 were continuously bred with two females per male for two weeks, or four estrous cycles, while being exposed according to their assigned group. The commercially available electronic cigarette refill pod used was Vuse Golden Tobacco, which contained 5% of nicotine, or $1/20^{th}$. The exposures started four days before the introduction of the male and lasted until the day before the delivery of the litter. To account for any differences that may be caused by stress on the animal, both groups were placed in a similar exposure chamber at the same time and were treated similarly. Four mating cycles were allowed in order to maximize the number of litters, and the male was removed once pregnancy was confirmed by a change in weight. Two exposure

sessions were conducted every day, with one in the morning and one in the afternoon, and the animals were switched in between. In order to account for any difference due to timing of exposure in the day, the mice were switched from one exposure session to the next on a day-to-day basis. A day of exposure occurred as follows: first, the females were weighed in a container and placed in an insect cage with a metal grid placed on top of the box. They were then brought to the exposure room, in which the machine was found, and placed inside one of the two exposure chambers: vaping exposed or sham depending on their assigned group. The machine was then started and consisted of a pump extracting 2 puffs a minute from the electronic cigarette. The battery of the electronic cigarette was replaced between each procedure and the pod was weighed before and after exposure to ensure that the machine worked properly. The animals were thus exposed for an hour, with a verification every 15 minutes to check for any sign of distress. After the hour of exposure, the electronic cigarette was unplugged from its pump, and the chambers were allowed 30 minutes to remove any e-vapor from the chambers. Then, the mice were removed from the chambers and brought back to their cage in the animal room. The next batch was then prepared.

The litters were weighed daily, and the sex were assigned with visual assessment a few days after birth. The pups were weaned 20 to 23 days after birth (PD20-23) and separated by sex. The offspring were then euthanized two days after weaning, PD22-25. On the morning of euthanization, two males and two females from each litter were selected. Those pups were fasted beginning at 4:45AM, two hours before the lights turned on in the animal room, for four hours in total. At this point, one male and one female were weighed and euthanized via carbon dioxide and cardiac puncture, and their blood was collected along with the liver, the spleen, the kidneys, the lungs, the brain, and the cecum which were harvested. The remaining offspring were refed

for two hours and euthanized in a similar way. The liver, kidney, and spleen were weighed before being placed in RNALater or 10% neutral buffered formalin, to conserve the RNA content of the cells and the integrity of the organ. The brain and part of the kidney and liver were flash frozen in liquid nitrogen. In this histological analysis, only fasted animals were analyzed, and 6 kidneys were sham males, 6 were sham females, 6 were vape females, and 5 were vape males, giving a total of 23 kidneys to be analyzed. The 6 sham males were siblings of the 6 sham females, while the 5 vape males were siblings of 5 of the vape females.

The kidney samples to be analyzed histologically were placed from neutral buffered formalin into ethanol the following day, and were sent to an external facility, to AML labs (St. Augustine, Florida), and were sectioned and stained using hemotoxilin and eosin stains. The images were taken with an iPhone SE from 2020, and the images were analyzed using ImageJ. Various measurements were obtained through ImageJ by surrounding the various features, including glomeruli, cortex and medulla, and calculating the area, finding the area of the glomeruli, the cortex area, the medulla area, and the number of glomeruli. The measurements were then standardized to cortex area to calculate the relative glomerular area and the density of glomeruli. The remaining measurements included the mean and the median area of the glomeruli on each slide. The median area of one of the slides was not calculated for issues with data collection. The slides were also observed under ImageJ to look for any sign of damage. Those measurements were then analyzed using 2-way ANOVA tests for interaction effects between two variables: sex and prenatal exposure. This test was run on GraphPad Prism. The graphs present in the data section were obtained through this software using either a t-test or a Two-way ANOVA test, and the mean and the standard error are shown.

## RESULTS

After four estrous cycles, not all females were pregnant, and not all females gave birth to a living litter. The weight of the offspring was significantly larger in the vaping group on PD2 ($p \leq 0.05$). The litter size was also observed to be lower in the vape group compared to the sham group ($p \leq 0.05$). The weights of the offspring normalized and by PD19, this difference was no longer observable (see Figure 1). At euthanization, the kidneys were observed as being proportionally larger in females than in males, and in females only, exposure to vaping resulted in a smaller kidney relative to body size (see Figure 2).

No obvious anomalies were visible on inspection of the cortex and medulla at both 10X and on inspection of the glomeruli at 40X magnification. No signs of edema nor fibrosis were found at 10X, and no sign of glomerular capillaries damage, nor of glomerular space was seen at 40X. Thus, when analyzed qualitatively, neither image showed any sign of obvious differences (see Figures 3 and 4).

Measurements were standardized to cortex area and included the average glomerular size (Figure 5), the median glomerular size (Figure 6), the density of glomeruli (Figure 7) and the glomerular area (Figure 8). No statistically significant difference was observed in any measures, but a trend was found of a sex-exposure interaction in the density of glomeruli and the glomerular area, in which only males seemed to have a lower density of glomeruli and relative glomerular area when exposed to vape.

## DISCUSSION

We report a limited though potentially interesting impact of prenatal ENDS exposure on reproductive outcomes. First, the litter size of the Vape dams was significantly smaller that the

Sham dams. The mechanism behind this reduction is unclear, as no investigation has been done regarding this issue, and a few hypotheses can explain this result: a reduction in fertility in the mother, an impairment in implantation in the uterus, or the death of embryo in utero or miscarriages. Further investigation will be required to know more about this reduction in litter size in dams having been exposed to electronic cigarettes.

This difference in litter size can account for the difference in weight between the Vape and the Sham group at birth. More nutrients have gone in each offspring born from the Vape group, resulting in them being more developed. There could also be an impact from vaping on the weight of the pup but given that cigarettes are known to result in reduction in weight at birth, this first hypothesis sounds more probable. This difference in weight disappeared by weaning. After weaning and euthanization, relative kidney weight was observed to be larger in female offspring, with a significant reduction in the Vape group compared to the Sham group. There was an interaction between exposure group and sex, as this reduction in kidney size due to exposure was only seen in females. The mechanism of this interaction was not determined and requires further investigation.

According to the Barker's and Brenner's hypotheses, the damage early on resulting from development may be minor and not observable, but results in epigenetic changes and alterations that may result in damage observable later in life. As expected from this hypothesis, no obvious histological damage was observed, but there may be minor alterations only observable through measurements in Vape offspring. These alterations include a reduction in glomerular size and number, which may result in the development of hypertension and CKD.

Thus, there is not enough data to prove that e-cigarette caused a reduction in kidney viability, but based on the data mentioned in Figures 2, 6, and 7, there was some evidence that in

both males and females, prenatal exposure to vaping altered the proper development of the kidney, albeit in a different way. In females, the kidney size itself was altered as the kidney was smaller relative to body size, despite the animals having the same weight, but the density of glomeruli did not change. In males, the relative size of the kidney was unaltered, but it appears that the density of glomeruli tended to be lower in the vaping group compared to the sham group. It is important to keep in mind that this difference was not significant, but there was a trend of an interaction between sex and exposure noted by the statistical analysis test ($p = 0.0974$ for number of glomeruli/cortex area ratio data; $p = 0.0896$ for glomerular area/cortex area ratio data). Thus, two measures of kidney structure exhibit a tendency to reduction and may impact kidney function in later life. Further investigation is needed to determine the mechanism of this interaction between sex and exposure group, and more details on the development of the glomeruli themselves.

**CONCLUSION**

In this study, the number of offspring was significantly lower in the group exposed to ENDS compared to the Sham group. The relative kidney weight was significantly higher in females relative to body size compared to males, and there was a reduction in relative kidney weight only seen in females due to the exposure to vaping. When analyzing the kidney histologically, no difference can be observed, but a trend appeared when comparing the number of glomeruli, and, as a result, the area of the glomeruli when normalized to cortex area. This trend was a reduction in glomeruli number and area only seen in males. Thus, it appears that vaping reduced the viability of the kidney in both males and females through a different pathway. In males, the glomeruli number seems to have been altered, and in females, the kidney size itself was altered. Further investigation is needed and should focus on the quality of the

glomeruli themselves, through measuring the number of nuclei and other metrics, and a biochemical assay of whether any pathway is altered in these kidneys. These pathways could be related to gluconeogenesis as the SIRT-1 and FXR pathways but could also be developmental. Further analysis will be performed on the kidneys to determine the developmental impact of this prenatal exposure, and the kidneys from 3-month-old littermates will also be analyzed.

**DATA AND FIGURES**

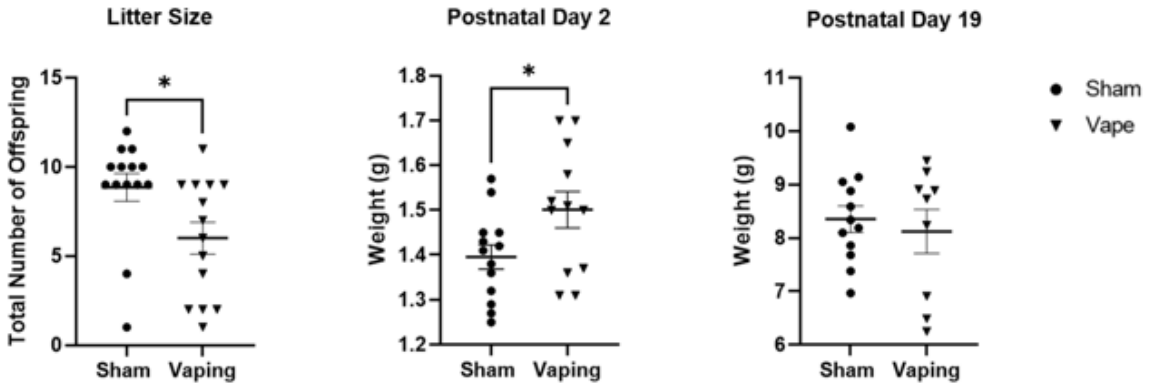
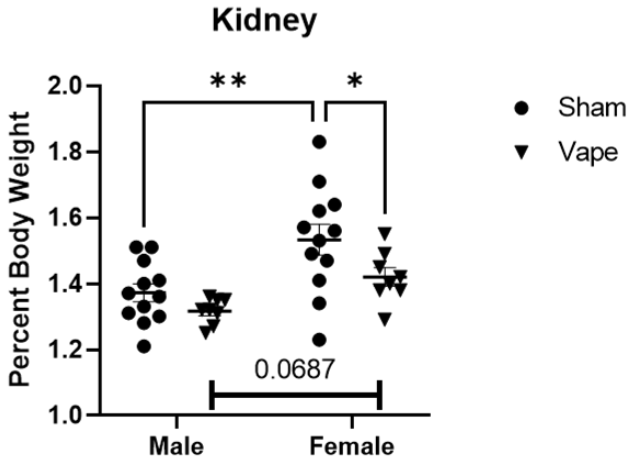


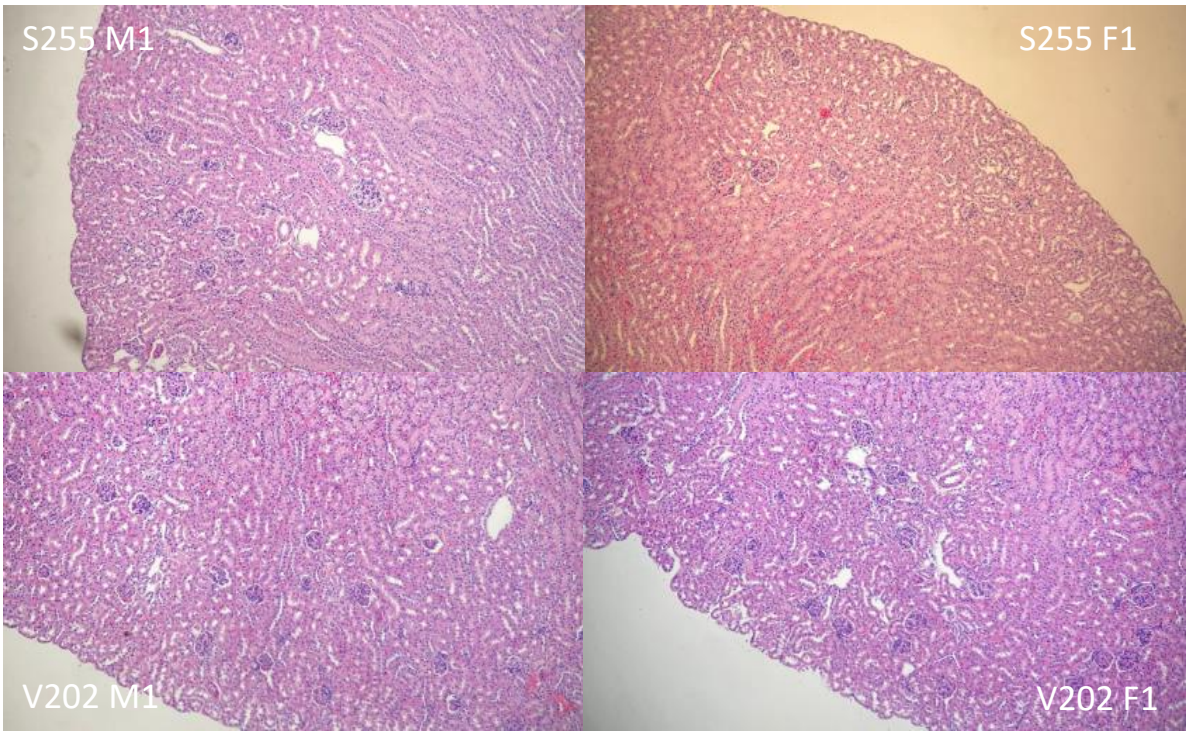

*Figure 1: Impact of prenatal vaping exposure on litter sizes and offspring weights.*

The average litter size (each data point represents a single litter) was lower in the Vape group compared to the Sham group, with a reduction by 2 offspring per litter noted. The average offspring weight (each data point represents a single litter offspring weight average) of the Vape group weighed more but returned to the average weight of Sham offspring by weaning (*$p \leq 0.05$).



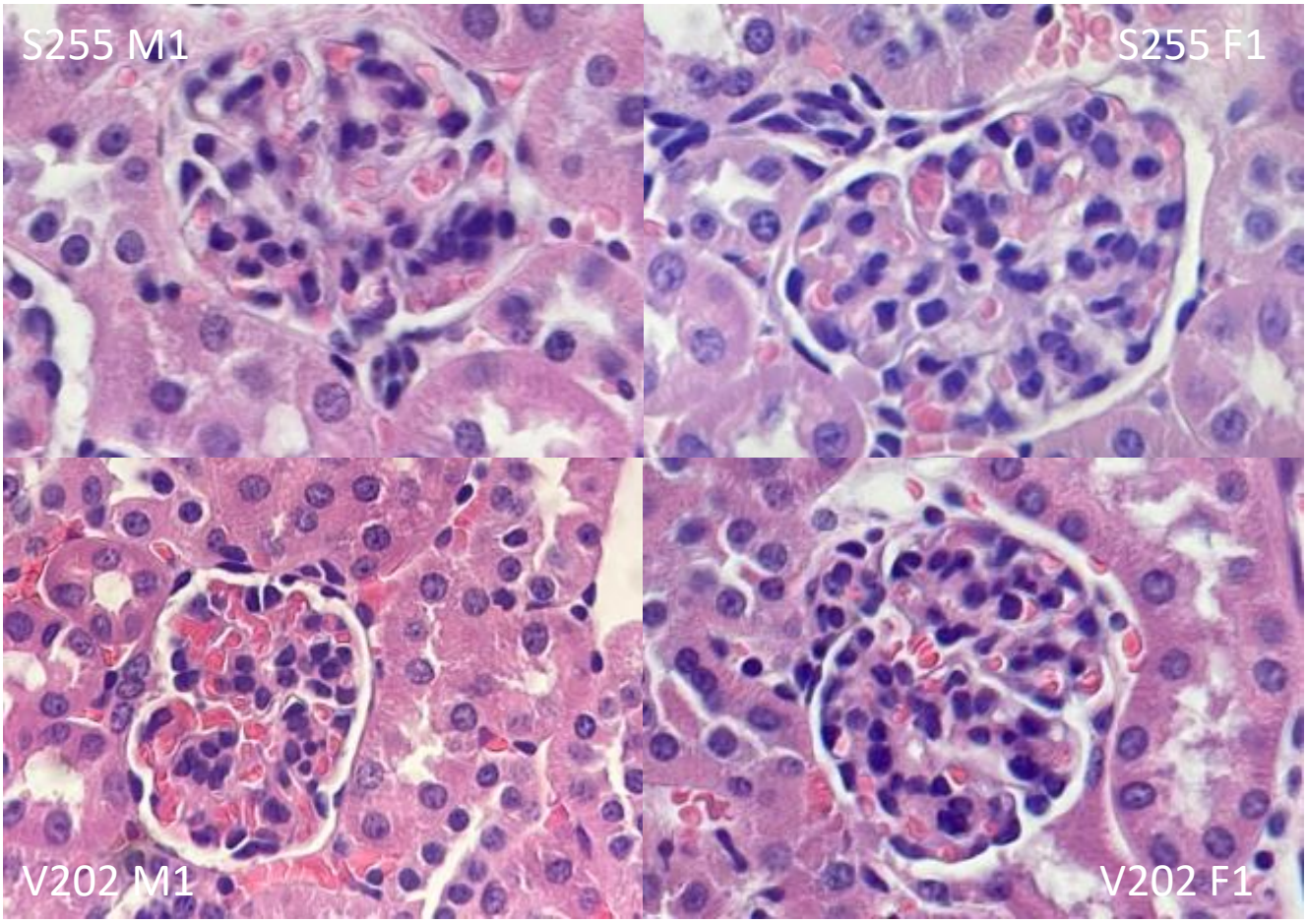

*<u>Figure 2:</u> Impact of prenatal vaping exposure and sex on relative kidney sizes at 3 weeks of age, just past the age of weaning.*

The relative kidney weight (each data point represents a single offspring) represented as percent of total body weight was larger in females compared to males in both gestational exposure groups (Sham and Vape). In female offspring only, the gestational exposure to Vape resulted in a smaller relative kidney weight (*$p \leq 0.05$; **$p \leq 0.001$).




*<u>Figure 3:</u> Histological comparison of kidney from Sham and Vape offspring at 3 weeks of age; (Sham male (top left), Sham female (top right), Vape male (bottom left), and Vape female (bottom right).*

There was no obvious sign of gross kidney damage in any of the sexes or groups, as expected since the offspring remained healthy. A single kidney from a single male and/or female offspring per litter was examined.




*<u>Figure 4:</u> Comparison of glomeruli based on gestational exposure and sex; Sham male (top left), Sham female (top right), Vape male (bottom left), and Vape female (bottom right).*

The glomeruli are center with the red marks inside the glomeruli showing the capillaries, and the space surrounding as the glomerular space. Outside of this space lies a thin basement membrane with elongated cells called the glomerular basement membrane. No obvious sign of damage was found in any of the examined glomeruli.

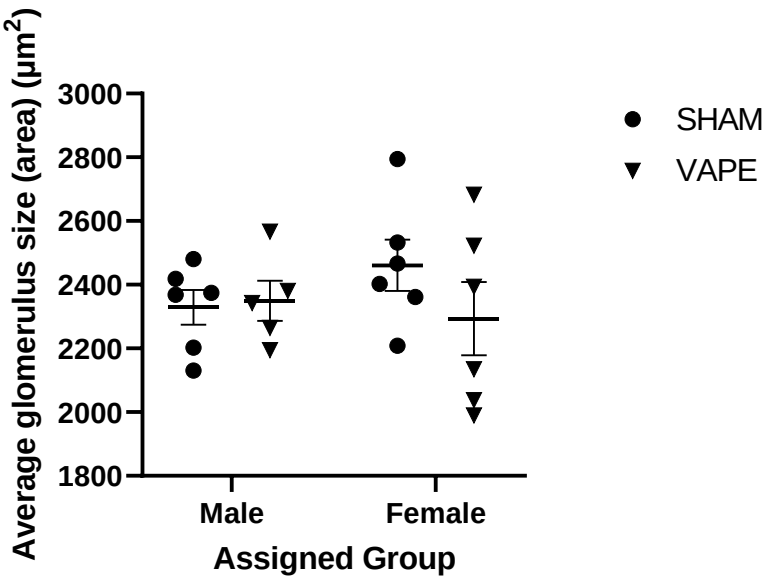
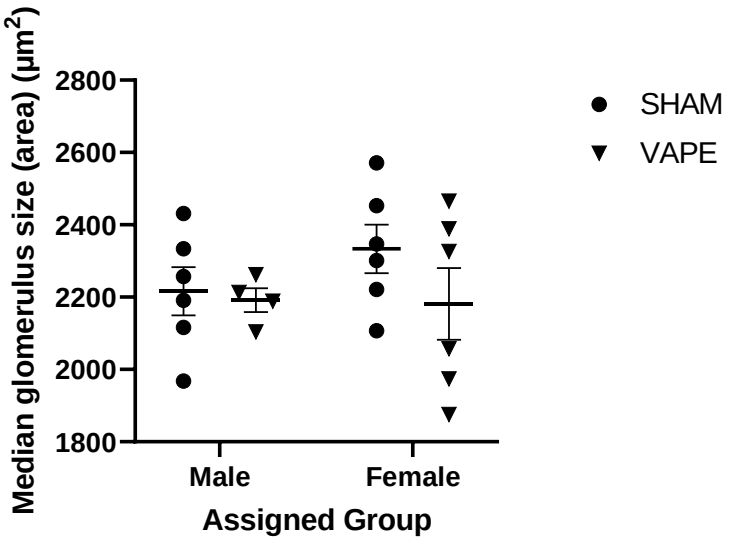


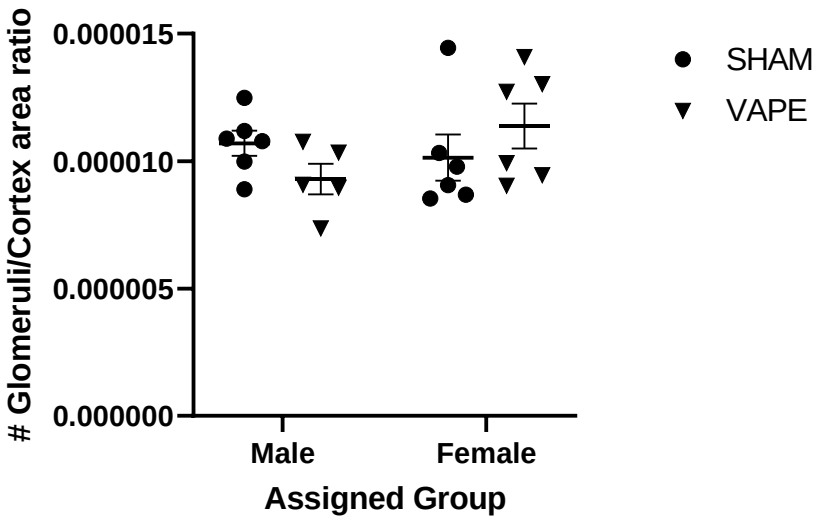

*Figure 5: Average glomerulus size based on gestational exposure and offspring sex.*

There was no evident difference in average glomerulus size (each data point represents a single kidney from a single offspring; single offspring per litter examined) in glomerular size with no interaction between sex and exposure.




*<u>Figure 6:</u> Median glomerulus size based on gestational exposure and offspring sex.*

No differences were noted in median glomerulus size in any group (each data point represents multiple glomerulus size measurements per histological slide for a single kidney from separate offspring; only 1 offspring per litter examined).




*<u>Figure 7:</u> Number of glomeruli normalized to cortex area sorted according to gestational exposure and offspring sex.*

There was a reduction in density of glomeruli in male Vape offspring as compared to male Sham offspring, with no impact of gestational exposure evident in female offspring. There was a trend of a sex-exposure interaction (2-way ANOVA; $p = 0.0974$).

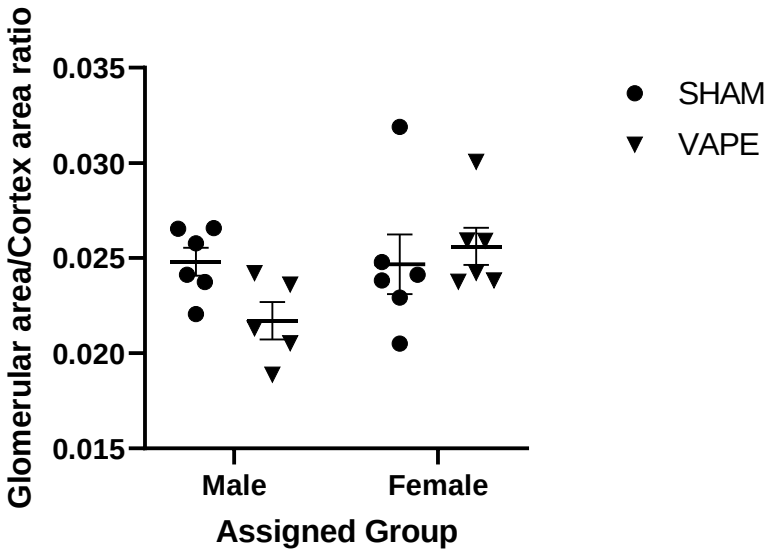



*Figure 8: Total glomerular area normalized to total cortex area sorted according to gestational exposure and offspring sex.*

There was a reduction in relative glomerular area found only in males Vape offspring with a trend noted for a sex-exposure interaction (2-way ANOVA; $p = 0.0896$).

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