COMPENSATORY GLOMERULOPATHY IN 3H1 BRACHYRRHINE MICE WITH GENETIC RENAL HYPOPLASIA IS EXACERBATED BY SALT TREATMENT

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ABSTRACT

The Brachyrrhine (Br/+) mice displays a mutation that directly affects the *six2* gene, and this results in premature development of the kidneys and nephrons. These mice exhibit renal hypoplasia which is characterized by a reduced kidney volume with a fewer number of nephrons. The purpose of this study is to compare the morphological differences between the 3H1 wild-type (Wt) and Br/+ mice subjected to salt loading. The specific aim of this project is to determine whether salt loading results in glomerulopathy in mice with heritable renal hypoplasia. A total of 24 3H1 mice, ranging in age from 12 to 20 weeks, were divided into four groups of 6 mice each: (1) Wt, no salt, (2) Wt, salt-treated, (3) Br/+, no salt and (4) Br/+, salt-treated. The salt-treated groups were given 2% NaCl solution as a sole source of their fluid intake for 5 days while the control animals were given distilled water. After the mice were perfusion-fixed at the end of the 5th day, the kidneys were removed and embedded in paraffin in preparation for histological sectioning. The sections were stained using H&E, and relevant sections were photographed using an Olympus BX41 light microscope. The sections were analyzed for the various stereological parameters and analyzed statistically. The final results showed significant differences between the Wt and the Br/+ in kidney volume, glomerular density/number, and glomerular surface area. The Wt mice were significantly larger in kidney volume, glomerular density, and glomerular number, while the salt-treated Br/+ were significantly larger in glomerular surface area. Results indicate that salt treatment worsens glomerulopathy in the Br/+ mice as a result of compensatory hyperfiltration.
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INTRODUCTION

Congenital renal hypoplasia is a common childhood condition characterized by a reduction in the number of nephrons and a small overall kidney size. This condition frequently leads to chronic kidney diseases (CKD) and end-stage renal disease (ESRD). The onset of adult CKD is mostly secondary to other diseases, such as, diabetes, obesity, and hypertension, but most of the childhood CKD below the age of twenty results from congenital causes. According to the North America Pediatric Renal Trials and Collaborative Studies (NAPRTCS), 18% of the pediatric renal cases involve genetic aplasia/hypoplasia/dysplasia of the kidneys, although there are differences between countries. Glomerulopathy is seen to increase with age, which implies that glomerular damage seen as a result of congenital defects, such as hypoplasia, takes longer to manifest itself. Among the different causes of CKD, the most common conditions leading to chronic renal failure (CRF) are hypoplasia/dysplasia and hereditary diseases in the 0-4 year age group. The Italkid Project of 2003 also reported that the leading causes of CRF were hypodysplasia associated with urinary tract malformations (53.6%). Although pediatric renal problems are a small percentage among the large adult CKD and end-stage renal disease (ESRD) patients, it is an important issue that needs to be continuously studied for prevention and treatment. The long-term goal of this research is to understand how glomerular number contributes to renal function, which could lead to better treatment options for pediatric renal hypoplasia patients to prevent subsequent CKD and renal failure. The specific aim of this study is to observe and report the effects of salt-loading on glomerular morphology in the Brachyrrhine (Br/+) mouse model with
congenital renal hypoplasia. A key to understanding mechanisms of renal hypoplasia resides in an understanding of nephron morphogenesis.

The role of six2 gene in kidney development

As with all other organs, the development of normal glomerular structure and function requires proper coordination and signaling between genes and kidney cells during the embryonic kidney development period.\textsuperscript{12,13,14} Although more work for clarification is needed, many studies have been undertaken to elucidate the signaling pathways behind nephrogenesis. Some of the earlier studies examined the development of nonmammalian vertebrates, such as, fish, frog, and chick embryos, but more recent studies have used mammalian vertebrates, such as, mice, rats, and humans in order to clarify the genes and pathways involved in nephrogenesis.\textsuperscript{3} It is important to note that all vertebrates have a common origin in terms of renal embryology, although environmental factors may alter the adult kidneys.

There are three types of mesoderm, and the type of mesoderm formed is determined by the exact location of the cell ingression into the primitive streak. Cells that enter through the midstreak region become the intermediate mesoderm, and this becomes vital to the development of the urinary system.\textsuperscript{9} A series of temporary and primitive kidney structures form from this tissue, the pronephros and mesonephros. The intermediate mesoderm gives rise to two lateral paired nephric ducts around E8.0, which eventually gives rise to the metanephros.\textsuperscript{45,46} The metanephric mesenchyme develops from the posterior intermediate mesoderm around E10.5.\textsuperscript{46} The ureteric bud also forms at
this time.\textsuperscript{21,22} The epithelial ureteric bud starts to invade the metanephric mesenchyme, and the interaction transforms the mesenchyme cells into epithelia.\textsuperscript{3,9} This invasion is induced by the GDNF (glial cell line-derived neurotrophic factor) ligand, and this is where branching morphogenesis begins. Mesenchymal cells aggregate at the tips of the ureteric buds and begin to transform into epithelia as more branching occurs.\textsuperscript{47} The mesenchymal aggregates then form renal vesicles, which develop two clefts to generate the s-shaped primitive nephron. The proximal end with the primary cleft develops into the glomerulus as endothelial cells enter the cleft.\textsuperscript{3} The activity of \textit{sin oculis 2 (six2)}, a transcription regulator, is needed to maintain a population of nephron progenitor for the generation of more nephrons and kidney growth.\textsuperscript{21,47} Mutation of \textit{six2} result in renal hypoplasia or agenesis. At E16.5, the \textit{six2}-nullizygous mice had a kidney approximately 65\% smaller than its wild-type littermate. The absence of \textit{six2} results in premature mesenchymal-epithelial transition that leads to ectopic renal vesicles, incomplete ureteric bud branching, and a lack of condensing mesenchyme.\textsuperscript{21} The \textit{six2} gene is needed to repress the inductive effects of Wnt-promoted nephrogenesis cascade, so that the mesenchymal progenitor pool will not be exhausted by premature nephrogenesis. Also, removal of the three \textit{hoxll} paralogs in mice resulted in complete failure of \textit{gdnf} and \textit{six2} expression in the area where metanephric mesenchyme forms.\textsuperscript{48} \textit{Hoxll} paralog expression in the intermediate mesodermal mesenchyme works with \textit{pax2} and \textit{eyal} to activate \textit{gdnf} and \textit{six2}. \textit{Pax2} mutations in mice are associated with apoptosis and renal hypoplasia.\textsuperscript{50} The absence of \textit{six2} promotes apoptosis of the mesenchymal progenitor population, which also adds to the development of a hypoplastic and defective kidney at
An x-ray irradiation-induced mutant mouse called Brachyrrhine (Br), originating from our lab, has been shown to exhibit defects in the development of the kidneys and craniofacial structures, which need proper induction signals during mesenchymal-epithelial transition (MET). Through various experiments done in our lab, we found that these mice display congenital renal hypoplasia that is heritable and is associated with haploinsufficient expression of *six2*. The *six2* gene, along with its interaction with other genes, is responsible for the proper development of the permanent, adult metanephros kidney. The Brachyrrhine kidneys are unable to properly serve its purpose of conservation, excretion, and electrolyte balance, because their hypoplastic kidney exposes them to structural and functional abnormalities. The hypoplastic kidneys, unable to sustain the organism, give rise to chronic renal disease which eventually leads to chronic renal failure and death. The homozygous Br/Br mutants die almost immediately upon birth due to the severity of their genetic defect, but the heterozygous Br/+ mutants survive to adulthood.

Chronic kidney disease (CKD) is a progressive disease that eventually leads to end-stage renal disease, which precedes renal failure. Based on previous studies done in our lab, the 3H1 Brachyrrhine mice display physiological features of CKD, such as, elevated mean arterial pressure, increased plasma creatinine, and dilute urine excretion. Morphological defects, such as, cysts and distended renal tubules were also found. In addition, previous stereological analysis done in our lab showed nephropathy, such as a smaller total kidney volume, less glomerular density/number, and a larger glomerular
surface area in the mutant mice compared to the wild-types. As a result of renal damage, the Brachyrrhine mice are also hypertensive, which worsens nephropathy. Once CKD is allowed to progress, the kidney progressively loses its critical function of glomerular filtration through structural damage. Chronic kidney disease, which eventually leads to renal failure, is defined as kidney damage or reduced glomerular filtration rate, and is a leading culprit of cardiovascular diseases and premature deaths. Hypertension and glomerular disease have been tightly related to each other as studies have found vascular calcification in the major vessels of most CKD patients. The Brachyrrhine mice exhibit hypertension and glomerulopathy as a result of less nephrons from renal hypoplasia.

A reduced number of nephrons at birth increases the risk of hypertension, which leads to glomerular abnormalities. Mammals born with a smaller kidney naturally have fewer nephrons corresponds to the Br mice. There is a similar disease seen in human beings called Oligomeganephronia. This disease produces renal hypoplasia with a reduced number of nephrons and eventually leads to glomerulosclerosis and end stage renal disease (ESRD). Renal ablation studies have shown development of proteinuria and glomerular sclerosis when renal mass is reduced. The glomerular capillary wall is damaged and proteins pass unrestricted through the barrier when injured glomeruli undergo compensatory hypertrophy. Glomerular hypertrophy increases pressure and distends the barrier. In another study, restoring renal mass by transplantation in rats lowered blood pressure and slowed the progression of glomerular injury in the remaining kidneys. Thus, reduced nephron number is expected to cause renal damage.

Aside from vascular calcification through uremic imbalance in the CKD
patients, hypertension is seen with a reduced number of glomeruli per kidney as glomerulopathy occurs. In order to compensate for lost nephron number, hyperfiltration results in the remaining surviving nephrons. Prolonged hyperfiltration by the glomeruli produces lesions which lead to glomeruli inflammation and subsequent CKD. Glomerular inflammation is accompanied by hypertrophy, which precedes injuries that eventually lead to chronic renal failure.

There are cases where the weight of the adult kidney is normal even though the nephron number is decreased, which implies compensatory renal hypertrophy. Correlation analysis done by our lab showed that the Br/+ mice with high plasma creatinine levels were more likely to have a high mean arterial pressure, positively correlating a reduced GFR with hypertension. The control and Br/+ mice were salt-treated in order to observe the morphological changes that occur in their kidneys.

Salt-treated kidney physiology

It has been well-known and widely studied throughout the years that a prolong diet high in salt worsens the effects of systemic hypertension, which can frequently lead to cardiovascular and renal diseases. In order to maintain normal regulation of arterial pressure, the balance between sodium and water intake and sodium and water excretion must occur. A normally functioning kidney is important in establishing extracellular fluid volume homeostasis. There are two sodium transporters, 1) thick ascending limb Na-K-2Cl cotransporter and 2) distal convoluted tubule Na-Cl cotransporter. These cotransporters were upregulated before and during the early phase of the development of
hypertension in rats. A study was reported where normotensive human beings were on normal and rich-sodium diets. The group with the high sodium diet had an increase in blood pressure parallel with an increase in glomerular filtration rate, suggesting a compensatory mechanism to counteract their inability to reabsorb sodium effectively. Defective tubular sodium handling has been associated with different genetic mutations and nutritional factors, such as a high salt diet.

In a salt-induced nephropathy experiment done with obese, hypertensive rats, a high-sodium diet of just 4 weeks exacerbated the symptoms of hypertension and renal disease. Already predisposed to renal diseases due to their obesity and hypertension, the high-salt diet injured the kidneys even further as proteinuria and renal lesions, such as, hypertrophic, sclerotic, and ischemic glomerular changes occurred. In another study, dietary salt loading as low as 4% of the diet resulted in chronic kidney disease symptoms, such as, proteinuria, albuminuria, and increase in mean arterial pressure, a decrease in GFR and renal plasma flow, and an increased renal vascular resistance and serum creatinine concentration. From the 4%, 6%, and 8% salt-treated groups, the 8% group had the highest arterial pressure elevation. Even among the lower salt-treated groups, severe glomerular dysfunction was seen as the afferent and efferent arteriolar resistances increased. This would eventually lead to glomerular injury, such as hypertrophy then sclerosis, commonly seen in chronic kidney diseases. In other experiments with a salt-restricted diet, the occurrence of glomerular hypertrophy, systemic hypertension, proteinuria, and tubular hypertrophy was reduced in rats with early-stage chronic renal failure. In contrast, salt-restriction diets were proven more effective in inhibiting the
symptoms of chronic renal failure than diuretics. The progression of glomerular injury by glomerular hypertrophy and tubular hypertrophy were inhibited by salt-restriction as expected.\textsuperscript{26}

The Brachyrrhine mutant mice are genetically predisposed to the development of chronic kidney disease and renal failure. The total cortex volume and glomerular number is expected to be less in the Br/\textsuperscript{+} mutants as this mutation results in renal hypoplasia. On the other hand, the glomeruli surface area of the mutants is expected to be greater than the Wt mice due to compensatory hyperfiltration resulting from reduced renal mass and nephron number. With the added stress of a high salt diet, the glomeruli surface area of the salt-treated adult heterozygous mutants will even be greater compared to the no-salt groups and Wt salt-treated group. Thus we expect that salt loading among Br mice would worsen glomerulopathy. Using stereology, this study will quantify the variation in the parameters to test hypotheses concerning the functioning of the wild-type, diseased/injured, and genetically altered kidneys.

\textit{Stereological analysis of glomerular morphology}

Three morphological parameters of interest, known as first-order stereological parameters, that will be studied are the following: 1) $V_{\text{ref}}$ – reference volume, specifically the cortex, 2) $N_v$ – glomerular density per area, and 3) $S_a$ – glomerular surface area. The glomerular number will be calculated by multiplying the reference volume by the glomerular density per area. These parameters will be compared among the following four mice groups: 1) Wt, no salt-treatment, 2) Wt, salt-treatment, 3) Br/\textsuperscript{+}, no salt-
treatment, and 4) Br⁻, salt-treatment. Using the stereology program, DisectorZ, the parameters will be calculated through an unbiased systematic random-sampling method.

The cortex volume was analyzed using the Cavalieri’s method which was first published in 1635 by the Italian mathematician Bonaventura Cavalieri. His principle showed that the volume of an arbitrarily shape object can be estimated in an unbiased manner from the product of the distance between planes and the sum of areas on systematic-random sections through the object. By repeating the estimate on several individuals from the population of interest, one obtains an estimate of the mean total volume for the reference space of interest. A point grid, or geometric probe, with a known area per point is used for estimating an area of interest. Gundersen and Jensen showed in 1987 that the combination of geometric probes and the Cavalieri estimator provides a theoretically unbiased and efficient method for estimating total volume from systematic-random sections.

The glomerular density per area was calculated using the dissector principle. In 1984, D.C. Sterio published the dissector principle, the first unbiased method for estimating the true number of objects in a given volume of tissue, \( N_v \). The dissector principle counts the number of cells using a 3-D probe. The dissector is a 3-D counting probe where the volume equals the area of the frame times the height. The unit is in cubic micrometers. This method counts “for the first time only” which means that each object within the probe is only counted at the initial intersection between the scanning plane and the object; at subsequent intersections, the object is not recounted. The dissector also has two sides of inclusion lines and two sides of exclusion lines which minimize bias. A grid
with counting frames is placed at random over a tissue section containing the reference space of biological interest. Objects to be counted in the tissue are separated by two thin adjacent sections, one section the reference and one the lookup. A \( Q^- \) is registered when an item to be counted is present in the frame on the reference section, but is absent on the lookup section. \( N_v \) is calculated by dividing the sum of the objects counted (\( Q^- \)) by the total volume of the dissector probes.

The surface area of the glomeruli was calculated using a grid with “virtual cycloids”\(^{53}\). Virtual cycloids curve according to a sine-weighted orientation, making theoretically unbiased estimates of total surface area on tissue sections cut at any convenient orientation. Biological tissues, such as the glomeruli, are originally 3D. When it is cut into 2D sections, the direction from which linear objects and surfaces are sectioned has a significant impact on the probability of intersections, and the resulting estimate can be biased. Thus, surface area are said to be anisotropic; their spatial orientations in 3D have unequal probabilities.\(^{53}\) The purpose of the cycloids is to transfer the differential sampling probability (\( x, y, z \) 3D-coordinates) to a 1-D line. Through complex mathematical predictions, cycloids ensure random probe-object intersections for the estimation of surface area.

The parameters of \( V_{\text{ref}}, N_v, \) and \( S_a \) in the four mice groups involved in this study were analyzed with the stereology program DisectorZ. This will provide the quantitative information needed to test our hypothesis. The purpose of this study as a whole is to analyze the quantitative and qualitative differences between the four mice groups using stereology techniques. The difference in kidney morphology between the Wt and Br/+
mutant mice and between the salt-treated and control groups will be analyzed using DisectorZ and Prism. It is expected that the Br/+ will show characteristics of renal hypoplasia, such as a reduced kidney volume and glomerular number. The hypothesis of this study is that the salt-treated Br/+ will have significantly larger glomerular surface area, compared to the Wt and no-salt, Br/+ groups, from compensatory glomerular hypertrophy resulting from renal hypoplasia.
MATERIALS AND METHODS

Animals

Adult male Br mice (20-30g) were housed and maintained on a 12h light/dark cycle with lights on at 6:00 A.M., with ambient temperature at 20-23°C. The experimental protocol was approved by the University of Hawaii Institutional Animal Care and Use Committee (IACUC). Investigators complied with the policies as prescribed in the United States Department of Agriculture Animal Welfare Act and the National Research Council’s Guide for the Care and Use of Laboratory Animals. Adequate measures were taken to minimize pain and discomfort in the animals throughout the study.

Twenty-four (n=24) total 3H1 adult mice, from ages 12 to 20 weeks, were used. They were divided into four groups of six mice each. The groups included: (1) Wt, no salt treatment, (2) Wt, salt-treated, (3) Br/+, no salt treatment and (4) Br/+, salt treatment. The mice were singly caged and allowed to acclimatize for 5 days prior to experimentation. Food and water were available ad libitum.

Hyperosmotic stimulation

The salt-treated mice were given 2% NaCl solution as a sole source of their fluid intake for 5 days. Control animals were provided with distilled water.
**Perfusion-fixation**

After the treatment, the mice were anesthetized using Avermectin (2.0 mg/10 g bodyweight) and perfusion-fixed transcardially with physiological saline, followed by phosphate-buffered 4% paraformaldehyde, pH 6.7 (Sigma).

**Kidney extraction and tissue preparation**

A total of 24 right kidneys, six from each mice group described above, were taken from the mice after perfusion-fixation. On day 1, the tissue is dissected in ice-cold phosphate-buffered saline (PBS) then rinsed again in fresh PBS. The kidneys were then placed in 4% paraformaldehyde in PBS and fixed for 12 hours with gentle rocking. On day 2, the kidneys were dehydrated using the following steps for 60-90 minutes: 1) 0.9% NaCl, 2) 30% EtOH in 0.9% NaCl, 3) 50% EtOH in 0.9% NaCl, 4) 70% EtOH in water, 5) 90% EtOH in water, and 6) 100% EtOH. The kidneys were then stored at -20°C until the “washing” and “embedding” steps.

**Washes**

The kidneys which were stored in ethanol at -20°C is washed in the following steps: (1) Three hours in 100% ethanol, (2) One hour in 100% Clear-rite (xylene substitute), and (3) Three hours in liquid paraffin at 60°C in an oven. The last 30 minutes of the last wash was done in a vacuum oven in order to prevent any bubbles from ruining the specimen.
Embedding

After the last step of washing, the kidneys were put into rectangular metal molds with the heated wax and left overnight to harden in preparation for sectioning.

Sectioning

All 24 blocks were trimmed downed on the sides and sliced at 10 micrometers thickness using the AO Spender “820” microtome. A ribbon of four consecutive sections were placed on a 40°C water bath for a few seconds then transferred to a slide. The slides were left to dry overnight on a dryer at 35°C so that the sections adhered to the slide during subsequent steps.

Staining

The dried slides were stained using Hematoxylin & Eosin in preparation for viewing under the microscope. They were placed into slide holders and dipped into the following chemicals: 100% clear-rite for 6 min., 100% ethanol for six min., 95% ethanol for 2 min., 85% ethanol for 2 min., water for 1 min., Gill’s Hematoxylin for 3 min., 0.1% HCl for 2 sec., water for 3 min., Alcoholic Eosin for 3 min., water for 1 min., 70% ethanol for 5 sec., 95% ethanol for 6 min., 100% ethanol for 6 min., and 100% clear-rite for six min. The slides were left to dry in the hood overnight then stored.
Photography

The sections were photographed using the Olympus BX41 microscope at 4x using the program Picture Frame.

Stereology analysis

The parameters of total cortex volume ($V_{ref}$), glomerular density per area ($N_v$), and glomerular surface area ($S_a$) were analyzed using the stereology program, DisectorZ, created by Dr. Scott Lozanoff and Mr. Ian Sharp. The glomerular number ($G_n$) was calculated by multiplying the cortex volume by the glomerular density per area ($V_{ref} \times N_v$).

Statistical Analysis with Prism

The quantitative relationship between the four mice groups mentioned above was analyzed using the statistics program Prism. One-way ANOVA (analysis of variance) was used to compare the numerical parameters of the four groups with a post-hoc Tukey Test for equivalence between individual means. One-way ANOVA is a statistical analysis used to test for differences among two or more independent groups. In this case, four different groups were analyzed. Graphs were generated to make quantitative comparisons of the morphological differences between the four groups.
RESULTS

The total cortex volume of both the salt-treated and control Wt was larger than the salt-treated and control Br/+ mutant mice, as expected, since Br/+ mice exhibit congenital renal hypoplasia. Both Wt groups were significantly larger than the Br/+ groups. The salt-treatment did not have an effect on the cortex parameters when the same genotype was compared. Genotype seems to be influential in determining cortical volume rather than the salt-treatment.

Figure 1 Comparison of the cortex parameter, or total reference volume, between the four groups. (*- significantly larger than the Br/+ S, ** significantly larger than both Br/+ groups) The Tukey's Test showed statistically significant differences between the wild-type and Br/+ regardless of their treatment method. Refer to Appendix A for raw data. (NS- no salt treatment, S- salt treatment)
The density of glomeruli per area was also significantly larger in both no-salt and salt-treated Wt compared to both the no-salt and salt-treated Br/+ groups, comparable to the results seen in the reference cortex volume above. This is also expected as hypoplastic kidneys correlate with a lower nephron number. The genotype seems to be influential in determining glomerular density rather than the salt-treatment.

![Comparison of Glomerular Density](image)

* Wt(ns) > Br/+(ns), Br/+ (s) p<0.001
** Wt(s) > Br/+(ns), Br/+ (s) p<0.001

Figure 2 Comparison of the glomerular density per area between the four mice groups. (*- significantly larger than the Br/+ groups, **- significantly larger than the Br/+ groups also) The Tukey's Test showed statistically significant differences between the wild-type and Br/+ regardless of their treatment method. Refer to Appendix B for raw data.
The average glomerular number was significantly larger in both no-salt and salt-treated Wt compared to both the no-salt and salt-treated Br/+ groups. This trend parallels those of the total reference volume and glomerular density per area as expected. Since the reference volume is smaller in the hypoplastic Br/+ kidneys, the glomerular density per area and number are also lower in these kidneys. As with the other parameters above, treatment did not have an effect on glomerular number. Genotype seems to be influential in determining glomeruli number rather than salt-treatment.

**Figure 3** Comparison of the glomerular numbers between the four mice groups. (*-significantly larger than the Br/+ groups, **- significantly larger than the Br/+ groups also) The Tukey's Test showed statistically significant differences between the wild-type and Br/+ regardless of their treatment method. Refer to Appendix C for raw data.
The glomerular surface area is significantly larger in the salt-treat Br/+ compared to both the salt-treated and no-salt Wt groups. The trend seen in this graph is inversely proportional to the other three parameters of volume, glomerular density, and glomerular number shown above. The salt-treated Br/+ has significantly greater glomerular surface area, showing that the treatment in combination with the mutation (genotype) has significant influence on this parameter.

Figure 4 Comparison of the glomerular surface area between the four mice groups. (*- The Br/+ S group had significantly larger glomerular Sa compared to both the Wt groups) The Tukey's Test showed statistically significant differences between the wild-type and Br/+ in correlation with both the genotype and the salt-treatment. Refer to Appendix D for raw data.
Figure 5. Qualitative Glomerular Morphology of a Wt Control Kidney. Compared to the Br/+ mice, the glomerular surface area is significantly smaller, especially in comparison to the salt-treated Br/+ kidneys. The yellow arrow is pointing at a glomerulus (Bowman’s Capule and glomerular tuft). See figure 4 and Appendix D for quantitative result comparisons and raw data. (Bar=100 μm)
Figure 6. Qualitative Glomerular Morphology of a Salt-Treated Wt Kidney. This is the morphological characteristic of a Wt control kidney. Compared to the Br/+ mice, the glomerular surface area is significantly smaller, especially in comparison to the salt-treated Br/+ kidneys. Although they are slightly larger than the no salt Wt control group seen in figure 8, the difference is insignificant. The arrow is pointing at a glomerulus. See figure 4 and Appendix D for quantitative result comparisons and raw data. (Bar=100 μm)
Figure 7. Qualitative Glomerular Morphology of a No-Salt Br/+ Kidney. This is the morphological characteristic of a no-salt Br/+ kidney. Compared to the wild-type mice, the glomerular surface area seems to be slightly larger, but the difference seen is not significant. This group is significantly smaller than the salt-treated Br/+ mice’s glomerular surface area. The process of glomerular damage seems to be slower and of lesser extent in this group compared to the salt-treated Br/+ . See figure 4 and Appendix D for quantitative result comparisons and raw data. (Bar=100 μm)
Figure 8. Salt-treated Br/+ Mice Kidney Morphology. The salt-treated Br/+ show significantly larger glomeruli surface area compared to the other three mice groups. Even between the two Br/+ groups, the salt-treated Br/+ glomeruli were significantly larger than the no-salt Br/++. The kidney anatomy seen in this figure shows extensively diseased morphology as the Bowman’s Capsule space is distended with overall glomerular hypertrophy and sclerosis. (A) This image seems to show a collapsing capillary tuft, spilling into the exterior part of the Bowman’s Capsule. (B) This image shows the large and abnormal ratio between the urinary space (white) and the capillary tuft (vascular aggregation inside the glomerulus). (C) This image also shows glomerular sclerosis as the contents and boundaries of the glomeruli disrupt. The glomeruli generally seem to be extremely large compared to the other three groups. (Bar=100 μm)
The wet kidney weight post-mortem showed an inverse relationship with the glomerular surface area shown above. The Wt groups were significantly heavier than the Br/+ positively correlating to the reference volume and glomerular number trend seen above. The wet kidneys also follow the same trend as the initial and final body weights of the mice. The treatment types did not have significant difference between salt and no salt, but the salt-treated Br/+ weighed less than the no salt Br/+. The genotype seems to be the main influence in determining kidney weight as with the reference volume and glomerular number.

Figure 9. Comparison of the right kidney wet weight post-mortem between the four mice groups. (*- significantly larger than both the Br/+ groups, **- significantly larger than the Br/+ S) The Tukey's Test showed statistically significant differences between the Wt and Br/+ in association with the genotype. See Appendix E for raw data.
The initial body weight is the weight of the mice before the experimentation started. Since it is before the salt-treatment, no large difference was observed in particular. There was significant difference between the weight of the mice between the Wt salt group and Br/+ salt group. The treatment is not a significant factor, but the genotype influence seems to play a main role in determining body weight as seen in the kidney weight, reference volume, and glomerular number trends above.

![Initial Body Weight](image)

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*Wt(s) > Br/+(s); p<0.05
```

Figure 10. Comparison of the initial body weight between the four mice groups. (*significantly larger than the Br/+ S group) The Tukey’s Test showed statistically significant differences between the salt-treated wild-type and Br/+ in association with the genotype. See Appendix F for raw data.
The final body weight was weighed on the 5th day of treatment. There is significant difference between the salt-treated Br/+ group and the other three groups. According to the results shown below, salt-treatment in conjunction with the mutation results in weight-loss. Compared to the initial weight, the final body weight of the salt-treated Br/+ shows weight loss which is most likely related to the salt treatment. This is seen as a result of a continuous cycle of dehydration as the salt solution ingested causes increased plasma osmolality. Ingestion of food induces further dehydration, so the mice lose their appetite.

![Figure 11 Comparison of the final body weight between the four mice groups. (* and ** and *** - significantly larger than the Br/+ S group) The Tukey's Test showed statistically significant differences between the salt-treated Br/+ and the three other groups. The Br/+ genotype along with salt treatment has significant influence. See Appendix G.](image)

* Wt(ns) > Br/+(s); p<0.001
** Wt(s) > Br/+(s); p<0.01
*** Br/+(ns) > Br/+(s); p<0.05
DISCUSSION

General

The heterozygous Brachyrrhine mice exhibit renal hypoplasia which produces smaller than normal kidneys with a reduced number of nephrons.\textsuperscript{52} The absence of \textit{six2} results in premature mesenchymal-epithelial transition that ultimately leads to an incomplete formation of the kidneys.\textsuperscript{21} When \textit{six2} is not properly expressed, premature nephrogenesis and apoptosis produces a hypoplastic kidney along with a reduced number of nephrons.\textsuperscript{21,47} The quantitative results seen from the stereological analysis coincides with what is expected in a congenital hypoplastic kidney. The quantitative results of reference volume, glomerular density, and glomerular number show similar trends. The Wt groups, regardless of treatment type, showed larger parameters compared to the Br/\textsuperscript{+} groups in the Vref, Nv, and Gn parameters. In these three parameters, the salt-treated group did not show significant difference from the control groups, showing that the result was mostly from genotype influence, not environmental. These results coincide with the description of congenital renal hypoplasia, as the kidney size and nephron number is determined before birth. Therefore, environmental factors, such as, salt-treatment that happened after birth, did not affect the volume and glomerular numbers. The wet weight of the kidneys and body weight was inversely related to the glomerular surface area between the four groups. This shows how the Br mutation affects the overall health status in general as weight loss is apparent as the glomerulopathy worsens. There are studies confirming a positive relationship between reduced glomerular/nephron number and a lower birth weight.\textsuperscript{55} The initial and final body weights of the mice generally parallels the
trend of the kidney weights, but the salt-treated Br/± showed considerable weight-loss. A possible explanation behind the weight-loss is that food exacerbates dehydration as the mice are continuously fed only salt solutions. Therefore, the mice discontinue food ingestion. The post-mortem wet kidney weight trend between the groups resembles the graphs of kidney volume, glomerular density, and glomerular number. This is expected as kidney weight decreases as kidney volume decreases. The nephrons are prematurely developed and fewer in number in renal hypoplasia, confirmed by the glomerular density/number results.

**Stereology Counting Method and Variations Seen in Species**

Transversely sectioned kidneys were analyzed with the Disector by creator Ian Sharp, and some of the parameters did not match the values of this study. Since the transverse sections had smaller references areas compared to the coronal sections used in this study, the smaller total reference (cortex) volume was expected. The glomerular surface area of his tranverse sections for the control matched the values reported here, but the salt-treated Br/± kidneys in this study showed considerably larger surface area compared to the no-salt Br/±. This is the result of the salt-treatment. Because of the smaller reference volume of the transversely cut sections, the glomerular number (Vref x Nv) was also smaller compared to the values of this study. A blind study was conducted with the same program by another operator, and the results closely corresponded with the values in this study. The value of Nv in the blind study differed by approximately 15%. Normally, large variations are seen among other species of animals. Even among the
same species, such as in *Homo sapiens*, there are great variations in glomerular number anywhere from around 300,000 to 2 million glomeruli per kidney.\(^{16}\) In a study done by Bertram et al., a considerable variation in the number of glomeruli was found, ranging from around 200,000 to 1,800,000.\(^{62}\) These larger variations in number were confirmed by another study from a range of 300,000 to 1,000,000 nephrons.\(^{61}\) The reason for the large variations is still debatable and not clearly stated in any of these studies. Genetic influences may alter the parameters, but secondary factors, such as, body weight, body size, and diseases may also play a large role in these wide ranges of glomeruli numbers. It is fair to say that genetic influences will play a large role in these wide variations, because most of the human nephrons are made during fetal development. The methodologies used, the investigator’s perspective, systematic errors, and small errors that occur during tissue processing may also play important roles in these variations seen.

**Glomerular Number Variations Among Mice**

As mentioned above, great variations in glomerular number exist even among the same species depending on the genetic makeup of the mice and the methodologies used for quantification. In one study, all the glomeruli of a newborn mice kidney were isolated using beads and magnets with 97% precision.\(^{64}\) It yielded 20,141 (±4699) glomeruli per kidney in a normal mice. Another similar study isolated total glomeruli by using acid and counted under phase microscopy with a counting chamber.\(^{65}\) The number came out to be 13,400 (±1,001). The glomerular number of our 3H1 wild-type mice range from around 30,000 to 60,000. The no-salt, Br\(^{+}\) range from around 12,000 to 20,000. The salt-treated,
Br/+ range from around 6700 to 12,000. Although the trend here is reasonable with the diseased Br/+ mutants having significantly smaller glomerular numbers compared to the Wt groups, the glomerular numbers are generally larger in this study compared to other studies mentioned above. Bonvalet et al. found that unilateral nephrectomy (or reduced renal mass) induced a compensatory overproduction of glomeruli in the remaining contralateral kidney of mice and rats. The glomeruli number increased from approximately 12,000(±3,000) to 14,000(±3,000). The larger numbers of glomeruli seen in this study may result from overall overproduction of glomeruli resulting from the smaller kidney volume of the bilateral hypoplasia. Most of the reduced renal mass studies involve surgical procedures done by an investigator or result secondary to other diseases, but our Br/+ mice have heritable reduced renal mass, or renal hypoplasia, which makes them unique. Other possibilities in explaining the number trend seen in our mice include the difference in methodologies used, subjective observations, systematic inconsistencies, and natural genetic influences between different mice strains.

Glomeruli Surface Area with Salt-Treatment in the Br/+:

As expected by our hypothesis, the glomerular surface area was significantly larger in the Br/+ groups, especially in combination with salt-treatment. The significantly larger value of the salt-treated Br/+ glomerular surface area compared to the other groups suggests that salt worsens compensatory glomerular hyperfiltration/hypertrophy and, therefore, contributes to CKD and subsequent renal failure. In one study, glomerular filtration was reduced by performing ureter ligation on nephritic mice with
glomerulosclerosis, and the glomerular damage was reversed along with suppressed hypertrophy.\textsuperscript{54} Maneuvers which reduce glomerular capillary pressure or flow were shown to limit glomerular injury.\textsuperscript{37} This suggests that compensatory hyperfiltration is the cause for glomerular hypertrophy and consequent damage seen in our mice.

Normally, when there is an increase in salt intake, there is a reduction in the activity of the renin-angiotensin-aldosterone system (increases salt retention) and an increase in atrial natriuretic peptides (increases salt excretion) in order to balance the salt intake with the excretion.\textsuperscript{7} An increase in salt intake also reduces the sympathetic nerve activity to the kidneys in order to increase the salt excreted to maintain mineral and fluid balance in the body.\textsuperscript{60} In a healthy kidney, a slight and transient increase in blood pressure is seen, but this is quickly compensated by autoregulation systems as the kidneys increases filtration to excrete the extra salt.\textsuperscript{4,5,6,7,8} Because the nephron numbers are high enough to sustain this change in filtration, very little if any damage is expected to be seen in the normal kidney. On the other hand, our Br/+ mice are prone to renal disease even before salt-treatment. This means that there are fewer nephrons and glomeruli initially, so the remaining nephrons must function at a higher level to filtrate the salt-load. This will inevitably lead to hyperfiltration in the remaining glomeruli and glomerular hypertrophy will become evident as the flow and pressure in the glomerular capillaries increase. The distended glomeruli will become insufficient as a filtration barrier and a higher blood pressure will be required to filter the load. Normally, as arterial blood pressure increase in the kidneys, more sodium and water will be excreted through pressure diuresis. In Br/+ mice, the compromised excretory function with the distended glomeruli hinders sufficient
salt and sodium excretion, resulting in an initial retention and expansion of blood volume in the body, followed by an increase in blood pressure in a futile attempt to alter the imbalance.

As a result of their smaller kidneys and lower nephron number, the heterozygous Brachyrrhine mice display physiological features of CKD, such as, elevated mean arterial pressure, increased plasma creatinine, and dilute urine excretion,\textsuperscript{24} which is consistent with their defective kidney morphology. The hypertrophied glomeruli, distended tubules, cysts, and hypoplastic kidneys all are characteristics of chronic kidney diseases that lead to renal failure.\textsuperscript{51} Damaged and obstructed tubule lumens may cause a backflow of filtrates onto the Bowman’s Capsule, contributing to the hypertrophy observed.\textsuperscript{67} In other studies, it is proposed that nephron deficit is the cause of essential hypertension in human beings,\textsuperscript{57,58} positively correlating the reduced nephron number to the high blood pressure seen in our mice. It has been found that a low nephron number hinders adequate excretion of dietary salt by pressure natriuresis at normal blood pressures, and increased renal perfusion pressures are needed to maintain salt and water homeostasis when there is an increase of dietary salt. Congenital nephron deficit, therefore, causes hypertension through autoregulatory mechanisms.\textsuperscript{55,56}
CONCLUSION

The parameters given by the stereological analysis of reference volume, glomerular density, glomerular number, and surface area supported the hypothesis proposed by this study. As a result of the haploinsufficient *six2* gene expression, the heterozygous Brachyrrhine mice displayed renal hypoplasia and reduced nephron/glomerular density. This caused the remaining glomeruli to undergo compensatory hyperfiltration, resulting in glomerular hypertrophy commonly seen in CKD patients. Because of their initially defective kidney morphology, the salt-treated group expressed the largest glomerular surface area and furthest extent of glomerular damage. Therefore, it can be concluded that inheritable renal hypoplasia in combination with salt-treatment exacerbates the initial glomerular deficiencies.
Appendix A

Vref (reference volumes of kidneys) of the Four Mice Groups

<table>
<thead>
<tr>
<th>Control Wt</th>
<th>Salt-Treated Wt</th>
<th>Control Br/+</th>
<th>Salt-Treated Br/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.756e10</td>
<td>1.2969e11</td>
<td>4.842e10</td>
<td>2.9465e10</td>
</tr>
<tr>
<td>1.2615e11</td>
<td>8.2801e10</td>
<td>6.305e10</td>
<td>4.89e10</td>
</tr>
<tr>
<td>1.2529e11</td>
<td>7.6596e10</td>
<td>7.05e10</td>
<td>5.792e10</td>
</tr>
<tr>
<td>6.094e10</td>
<td>7.5152e10</td>
<td>7.2009e10</td>
<td>7.1226e10</td>
</tr>
<tr>
<td>7.9812e10</td>
<td>1.1734e11</td>
<td>7.2186e10</td>
<td>2.9182e10</td>
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<tr>
<td>7.217e10</td>
<td>1.4722e11</td>
<td>6.4396e10</td>
<td>3.5736e10</td>
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</table>

*measurements in μm³

Appendix B

Nv (glomerular density per area) of the Four Mice Groups

<table>
<thead>
<tr>
<th>Control Wt</th>
<th>Salt-Treated Wt</th>
<th>Control Br/+</th>
<th>Salt-Treated Br/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.166e-7</td>
<td>4.2777e-7</td>
<td>2.527e-7</td>
<td>2.9166e-7</td>
</tr>
<tr>
<td>4.4822e-7</td>
<td>6.3333e-7</td>
<td>2.667e-7</td>
<td>1.9e-7</td>
</tr>
<tr>
<td>4.8611e-7</td>
<td>5.7916e-7</td>
<td>2.778e-7</td>
<td>2.333e-7</td>
</tr>
<tr>
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<td>2.8746e-7</td>
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<tr>
<td>4.6388e-7</td>
<td>4.7083e-7</td>
<td>2.4305e-7</td>
<td>2.3055e-7</td>
</tr>
<tr>
<td>4.25e-7</td>
<td>4.3055e-7</td>
<td>3.0135e-7</td>
<td>3.1944e-7</td>
</tr>
</tbody>
</table>

*measurements in number of glomeruli per area in μm³
Appendix C

Gn (glomerular number) of the Four Mice Groups

<table>
<thead>
<tr>
<th>Control Wt</th>
<th>Salt-Treated Wt</th>
<th>Control Br/+</th>
<th>Salt-Treated Br/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>50399.5</td>
<td>55477.49</td>
<td>12235.73</td>
<td>8593.76</td>
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<td>56542.95</td>
<td>52440.36</td>
<td>16815.44</td>
<td>9291</td>
</tr>
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<td>60904.72</td>
<td>44361.34</td>
<td>19584.9</td>
<td>13512.74</td>
</tr>
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<td>39775.54</td>
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<td>20699.71</td>
<td>20655.54</td>
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<td>37023.19</td>
<td>55247.19</td>
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<td>6727.91</td>
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<tr>
<td>30672.25</td>
<td>63385.57</td>
<td>19405.73</td>
<td>11415.51</td>
</tr>
</tbody>
</table>

*no units

Appendix D

Sn (glomerular surface area) of the Four Mice Groups

<table>
<thead>
<tr>
<th>Control Wt</th>
<th>Salt-Treated Wt</th>
<th>Control Br/+</th>
<th>Salt-Treated Br/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>6750.46</td>
<td>13009.31</td>
<td>13157.5</td>
<td>17333.91</td>
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<tr>
<td>10308.02</td>
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</tr>
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<td>11442.45</td>
<td>11245.71</td>
<td>13462.9</td>
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<td>8966.24</td>
<td>11138.95</td>
<td>13387.4</td>
<td>18961.81</td>
</tr>
<tr>
<td>12030.01</td>
<td>12620.61</td>
<td>22764.3</td>
<td>33034.01</td>
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<td>11543.01</td>
<td>11155.01</td>
<td>17743.1</td>
<td>18243.05</td>
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</table>

*measurements in μm²
Appendix E

Wet Kidney Weight Post-Mortem of the Four Mice Groups

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<th>Salt-Treated Wt</th>
<th>Control Br/+</th>
<th>Salt-Treated Br/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.303</td>
<td>0.255</td>
<td>0.152</td>
<td>0.131</td>
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<tr>
<td>0.254</td>
<td>0.198</td>
<td>0.193</td>
<td>0.128</td>
</tr>
<tr>
<td>0.286</td>
<td>0.286</td>
<td>0.191</td>
<td>0.172</td>
</tr>
<tr>
<td>0.303</td>
<td>0.329</td>
<td>0.18</td>
<td>0.202</td>
</tr>
<tr>
<td>0.46</td>
<td>0.294</td>
<td>0.251</td>
<td>0.084</td>
</tr>
<tr>
<td>0.165</td>
<td>0.318</td>
<td>0.175</td>
<td>0.149</td>
</tr>
</tbody>
</table>

*measurements in grams

Appendix F

Initial Body Weight (before experiment) of the Four Mice Groups

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<th>Control Wt</th>
<th>Salt-Treated Wt</th>
<th>Control Br/+</th>
<th>Salt-Treated Br/+</th>
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</thead>
<tbody>
<tr>
<td>28.77</td>
<td>24.61</td>
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<tr>
<td>26.31</td>
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<td>22.74</td>
</tr>
<tr>
<td>27.48</td>
<td>35.15</td>
<td>25.52</td>
<td>23.99</td>
</tr>
<tr>
<td>40.06</td>
<td>28.08</td>
<td>24.29</td>
<td>18.26</td>
</tr>
<tr>
<td>21.41</td>
<td>34.32</td>
<td>28.41</td>
<td>22.51</td>
</tr>
</tbody>
</table>

*measurements in grams
Appendix G

Final Body Weight (Day 5) of the Four Mice Groups

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<tr>
<th>Control Wt</th>
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<th>Control Br/+</th>
<th>Salt-Treated Br/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>27.95</td>
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<td>19.84</td>
<td>18.87</td>
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<tr>
<td>26.56</td>
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<td>27.68</td>
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<td>23.03</td>
<td>32.99</td>
<td>28.23</td>
<td>19.53</td>
</tr>
</tbody>
</table>

*measurements in grams
LITERATURE CITED


25. Lozanoff S, Johnston J, Ma W, and Jourdan-Le Saux C. Immunohistochemical localization of pax2 and associated proteins in the developing kidney of mice with renal


62. Hoy WE, Douglas-Denton RN, Hughson MD, Cass A, Johnson K, and Bertram JF. A


