ASSOCIATION BETWEEN CONGENITAL NEPHRON DEFICIT AND
SUSCEPTIBILITY TO DIABETIC NEPHROPATHY AND HYPERTENSION

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Abstract

Diabetic nephropathy (DN) is the most prevalent and severe complication of diabetes, affecting up to one-third of diabetics. Normally, it takes many years to develop DN since the first diagnosis of diabetes; however, a subset of diabetic population develops DN at an early stage, independent of diabetic control. Therefore, development of a mechanism to identify patients that have higher risk of rapid renal deterioration is essential. It has long been speculated that individuals who are born with low nephron numbers are more susceptible to the development of hypertension and acquired renal disease later in life. In humans, heterozygous mutation of the PAX2 gene are associated with reduced congenital nephron number and progressive renal insufficiency in childhood. In mice, heterozygous Pax2 mutations were shown to reduce nephron number by interfering with branching morphogenesis of the ureteric bud (UB). Therefore, we postulated that diabetic individuals with suboptimal nephron number will exhibit accelerated progression of DN.

To establish proof of concept, we developed a mouse model by crossing mice with heterozygous null Pax2 mutation (Pax2<sup>1Neu</sup>) and type I diabetic mice (Ins2<sup>2Akita</sup>), carrying a heterozygous mutation in the insulin2 gene. We demonstrate that mice with both Pax2 and Ins2 develop hypertension and albuminuria, early signs of DN, before any other genotype littermates with only one of the mutations or none. We further validated the effect of reduced nephron number on early manifestation of DN by rescuing the reduced nephron number caused by Pax2 mutation.

Furthermore, we investigated whether common polymorphisms of PAX2 gene associated with subtle renal hypoplasia result in early manifestation of DN. We tested the association
between these $PAX2$ SNPs and markers of DN in diabetic cohort who have been diagnosed with diabetes for 15 years or less. We demonstrated a significant association between $PAX2$ SNPs in diabetic patients and onset of albuminuria, the earliest marker of DN. $PAX2$ SNPs did not increase the likelihood of diabetic patients to develop hypertension and to exhibit progressive loss of GFR.

In summary, these observations suggest that congenital nephron deficit accelerates the progression of DN.
**Resume**

La néphropathie diabétique (DN) est la complication la plus fréquente et la plus sévère du diabète, touchant jusqu'à un tiers des diabétiques. Normalement, il faut plusieurs années pour développer le DN depuis le premier diagnostic de diabète; Cependant, un sous-ensemble de la population diabétique développe DN à un stade précoce, indépendant du contrôle diabétique. Par conséquent, le développement d'un mécanisme permettant d'identifier les patients présentant un risque plus élevé de détérioration rénale rapide est essentiel. Il a longtemps été spéculé que les individus qui sont nés avec de faibles nombres de néphrons sont plus sensibles au développement de l'hypertension et de la maladie rénale acquise plus tard dans la vie. Chez l'homme, la mutation hétérozygote du gène PAX2 est associée à un nombre réduit de néphrons congénitaux et à une insuffisance rénale progressive chez l'enfant. Chez la souris, il a été montré que les mutations hétérozygotes de Pax2 réduisent le nombre de néphrons en interférant avec la morphogenèse ramifiée du bourgeon urétéral (UB). Par conséquent, nous avons postulé que les individus diabétiques avec un nombre de néphrons sous-optimal présenteront une progression accélérée de DN.

Pour établir la preuve de concept, nous avons développé un modèle murin en croisant des souris avec une mutation Pax2 nulle hétérozygote (Pax21Neu) et des souris diabétiques de type I (Ins2Akita), portant une mutation hétérozygote dans le gène insulin2. Nous démontrons que les souris présentant à la fois Pax2 et Ins2 développent une hypertension et une albuminurie, signes précoces de DN, avant tout autre porteur de génotype ayant une seule mutation ou aucune. Nous avons également validé l'effet du nombre réduit de néphrons sur la manifestation précoce de DN en récupérant le nombre réduit de néphrons provoqué par la mutation Pax2.
De plus, nous avons étudié si les polymorphismes communs du gène PAX2 associés à une hypoplasie rénale subtile entraînent une manifestation précoce de DN. Nous avons testé l'association entre ces SNP PAX2 et les marqueurs de DN chez les diabétiques ayant reçu un diagnostic de diabète depuis 15 ans ou moins. Nous avons démontré une association significative entre les SNPs PAX2 chez les patients diabétiques et le début de l'albuminurie, le premier marqueur de DN. Les SNP de PAX2 n'augmentaient pas la probabilité que les patients diabétiques développent une hypertension et présentent une perte progressive de DFG.

En résumé, ces observations suggèrent que le déficit néphron congénital accélère la progression de DN.
Contribution of Authors

This thesis is written in accordance with the guidelines provided by Faculty of Graduate and Postdoctoral Studies at McGill University. The research reported in this thesis was carried out and all the results presented in the thesis was generated by Sangmi Sarah Park under the guidance of Dr. Paul Goodyer. Dr. Murielle Akpa helped with processing of mouse urine samples for albumin and creatinine assays, and mouse serum samples for cystatin C assay.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACR</td>
<td>Albumin-to-creatinine ratio</td>
</tr>
<tr>
<td>CM</td>
<td>Cap mesenchyme</td>
</tr>
<tr>
<td>DN</td>
<td>Diabetic nephropathy</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>GBM</td>
<td>Glomerular basement membrane</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
</tr>
<tr>
<td>htSNP</td>
<td>Haplotype-tagging single-nucleotide polymorphism</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-to-epithelial transition</td>
</tr>
<tr>
<td>MM</td>
<td>Metanephric mesenchyme</td>
</tr>
<tr>
<td>PAX</td>
<td>Paired box</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RCS</td>
<td>Renal-coloboma syndrome</td>
</tr>
<tr>
<td>SNP</td>
<td>Single-nucleotide polymorphism</td>
</tr>
<tr>
<td>TBM</td>
<td>Tubular basement membrane</td>
</tr>
<tr>
<td>UAE</td>
<td>Urinary albumin excretion</td>
</tr>
<tr>
<td>UB</td>
<td>Ureteric bud</td>
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<tr>
<td>WD</td>
<td>Wolffian duct</td>
</tr>
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</table>
CHAPTER I: Introduction
1.1 Overview and Rationale for the Research

The kidney is an important organ that plays a critical role in homeostasis by regulating body fluid composition and excretion of metabolic waste products. The mammalian kidney and its intricate structures in particular, need to be appreciated as it has gone through various evolutionary modifications that permitted the adaptation to the highly specialized environment since the first excretory organ in primitive fish (Natochin, 1996; Romagnani, Lasagni, & Remuzzi, 2013; Smith, 1953). The adaptation enabled the ability to reabsorb and conserve water and hence, the concentration of urine, which ultimately allowed the transition from life in aqueous environment to life in dry terrestrial environment. Despite the fact that the kidney has evolved to be structurally different across various vertebrate classes, it is important to note that the process of the development of the kidney and the basic structure of the nephron, the functional unit of the kidney, remain highly conserved (Dressler, 2006; Schedl, 2007). Nephrons consist of glomeruli, which are responsible for filtering the blood to remove waste, and tubular structures, which facilitates reabsorption and excretion of solutes before the urine is transmitted to the collecting duct to be emptied into the bladder (Davidson, 2008; Krause, Rak-Raszewska, Pietila, Quaggin, & Vainio, 2015; Murawski, Maina, & Gupta, 2010; Takasato & Little, 2015).

Aside from the structural differences, the major difference that separates the mammalian renal system from that of other vertebrate classes is the inability to facilitate nephron neogenesis once nephron maturation is completed at around birth, whereas other vertebrate classes, such as fish, amphibians and reptiles are able to generate new nephrons throughout their lives. Fortunately, the newly acquired structure of the mammalian kidney allowed the number of nephrons to increase considerably compared to other animal classes. In fact, mammals generally generate excessive number of nephrons during embryonic development, thus the ability to
generate new nephrons became no longer crucial in the mammalian renal system (Dantzler & Braun, 1980; Luyckx & Brenner, 2010; Quigley, 2012).

Nephrogenesis is initiated during early stages of embryogenesis when the ureteric bud (UB) arises from the Wolffian duct, penetrates the metanephric mesenchyme (MM) and begins branching (Dziarmaga, Eccles, & Goodyer, 2006; Little et al., 2007). A subset of the MM condenses around the tip of each branch to promote continued UB branching and eventually leads to the formation of nephrons (Dziarmaga et al., 2006; Short et al., 2014). Hence, the final nephron number and the functional capacity of the kidney are determined by the extent of UB branching during fetal development. In humans, nephrogenesis ceases at 36 weeks of gestation, at which point the renal progenitor population exhausts and the nephron number is established (Hinchliffe, Sargent, Howard, Chan, & van Velzen, 1991). In mice, nephron development persists for the first few postnatal days, but nephrogenesis does not occur once the nephron progenitor population is lost past that point (Hartman, Lai, & Patterson, 2007; Rumballe et al., 2011).

Interestingly, nephron number varies greatly in humans, ranging from 210,000 to 2,700,000 according to a recent autopsy study of 420 human kidneys (Bertram, Douglas-Denton, Diouf, Hughson, & Hoy, 2011). Although the wide variance in nephron number may be perceived as a variability in nephron endowment with no significant clinical relevance, Brenner et al. proposed that individuals born with suboptimal nephron numbers are at higher risk for developing essential hypertension due to reduction in filtration surface area (Brenner, Garcia, & Anderson, 1988). Furthermore, Brenner et al. also suggested that since congenital nephron deficit will lead to compensatory hyperfiltration, the glomerulus will expand in order to match its filtration capacity to the body’s demand, eventually leading to glomerular sclerosis and putting
those individuals endowed with reduced nephrons at a higher risk of progressive renal damage at the onset of acquired renal disease (Brenner et al., 1988; Luyckx, Shukha, & Brenner, 2011). Likewise, nephron number may contribute to susceptibility of subsets of diabetics to develop renal disease (Brenner et al., 1988). Since Brenner’s proposition, several studies have reported the association between nephron number and adult diseases (Barker, Osmond, & Law, 1989; Brenner, 1994; Brenner & Mackenzie, 1997; G. Keller, Zimmer, Mall, Ritz, & Amann, 2003). In particular, Keller et al. demonstrated that individuals with essential hypertension had 46% fewer nephrons and 133% higher mean glomerular volume compared to normotensive age-matched individuals (G. Keller et al., 2003), supporting Brenner’s hypothesis.

Factors that determine the final nephron number during kidney development are unknown. However, PAX2, a developmental transcription factor, has been shown to play an important role in nephrogenesis by mediating UB branching. Indeed, individuals with heterozygous PAX2 mutations, as in the case for renal-coloboma syndrome (RCS), are diagnosed with congenital deficit of nephrons and renal hypoplasia (Sanyanusin, McNoe, Sullivan, Weaver, & Eccles, 1995). Our lab has previously demonstrated that PAX2 suppresses apoptosis in the UB during nephrogenesis and that loss of PAX2 anti-apoptotic effect reduces the extent of UB branching in embryonic kidney (Clark, Dziarmaga, Eccles, & Goodyer, 2004; Porteous et al., 2000; Torban, Eccles, Favor, & Goodyer, 2000). We also discovered common variants of the PAX2 gene, which were associated with 10% reduction in renal volume, a surrogate for nephron number, in the newborn cohort (Quinlan et al., 2007).

There is a wide variation in the susceptibility to nephropathy in human diabetic patients. We propose that individuals who are born with reduced nephron numbers may represent those patients that are at a higher risk of accelerated progression to renal damage. The primary
objective of my research project was to determine whether congenital nephron deficit results in worse outcomes in diabetic mice. The second objective of my research was to investigate the association between common variants of the PAX2 gene and early onset of diabetes in the human diabetic cohort.

### 1.2 Kidney Development and Nephrogenesis

The complex structures in the mammalian kidney is the result of multiple stages of evolution driven by the change in living environment. The main processes involved in the kidney development remain highly conserved across phyletic groups; however, the kidney in amniotes is unique in that three distinct kidney types arise in series of successive phases during embryogenesis. The early two forms of the kidney, pronephros and mesonephros, are transient organs that subsequently degenerate in amniotes but are required for the development of the mature kidney, metanephros (Davidson, 2008; Krause et al., 2015; Moritz, Wintour, Black, Bertram, & Caruana, 2008). The development of metanephric kidney is initiated when the ureteric bud (UB), derived from the Wolffian duct (WD), invades a cluster of cells referred to as the metanephric mesenchyme (MM). The MM condense and aggregate at the tip of the UB, forming the cap mesenchyme (CM), a population of nephron progenitors that proliferate and differentiate into glomerular and tubular epithelial cells to induce UB branching and nephron formation. Multiple sequential bifurcation events take place at the UB tip, generating nephrons arranged in arcades, which gives the mature kidney its arborized morphology consisting of collecting ducts connected to nephrons (Shah, Sampogna, Sakurai, Bush, & Nigam, 2004). These steps repeat throughout kidney development until nephrogenesis comes to a completion at 36 weeks of gestation in humans and first few postnatal days in mice (Hartman et al., 2007; Hinchliffe et al., 1991; Rumballe et al., 2011). Once nephrogenesis ceases, generation of new
nephrons is no longer possible. The extent of UB branching during fetal development therefore, is an important determinant of the final nephron number and the functional capacity of the kidney.

1.3 Clinical Importance of Nephron Number

Nephron number varies greatly between individuals in the human population, ranging from 210,000 to 2,700,000 according to a recent autopsy study of 420 human kidneys (Bertram et al., 2011). This variation was generally believed to be a consequence of variability in nephron endowment determined during embryogenesis with no clinical significance, until Brenner et al. proposed the idea that congenital nephron deficit may be associated with susceptibility to the development of hypertension and acquired renal disease in adulthood (Brenner et al., 1988). Brenner reasoned that less nephrons will mean reduced filtration surface area, which will contribute to renal sodium retention and hence, the development of essential hypertension as a compensatory mechanism. Following this systemic hypertension, the blood pressure in capillaries of the glomerulus will also increase and cause renal hypertrophy and damage, which will further reduce filtration surface area and perpetuate a vicious cycle. Those individuals born with nephron number at the lower end of the spectrum will then also be at a higher risk of progressive renal disease following initial renal injury due to reduced ability to sustain renal function compared to those individuals born with relatively high nephron numbers.

In line with Brenner’s hypothesis, Keller et al. reported autopsy studies on 10 individuals with hypertension and 10 age-matched normotensive individuals, which demonstrated 46% fewer nephrons and 133% higher mean glomerular volume in hypertensive patients compared to the normotensive controls (G. Keller et al., 2003). Bertram et al. also investigated the association between nephron number and blood pressure and observed inverse relationship between blood
pressure and nephron number in adult white Americans and Australian Aborigines (Hoy, Hughson, Singh, Douglas-Denton, & Bertram, 2006; Hughson, Douglas-Denton, Bertram, & Hoy, 2006). In addition, higher mean glomerular volume was observed in hypertensive African Americans compared to normotensive individuals (Hughson, Hoy, Douglas-Denton, Zimanyi, & Bertram, 2011), and in Caucasians with low nephron numbers compared to individuals with higher nephron numbers (Zimanyi et al., 2009). Few studies also reported that nephron number, was associated with renal pathology and measures of chronic kidney disease. Increased number of glomeruli with glomerulosclerosis and cortical fibrosis was observed in individuals with low nephron numbers (Douglas-Denton, McNamara, Hoy, Hughson, & Bertram, 2006; McNamara et al., 2008) and individuals with low birth weight, a surrogate for nephron number, exhibited increased rates of microalbuminuria, end-stage renal disease, focal segmental glomerular sclerosis and reduced glomerular filtration rate (Hallan et al., 2008; Hodgin, Rasoulpour, Markowitz, & D'Agati, 2009; Hoy, Rees, Kile, Mathews, & Wang, 1999; Keijzer-Veen et al., 2005; Lackland, Bendall, Osmond, Egan, & Barker, 2000; Vikse, Irgens, Leivestad, Hallan, & Iversen, 2008). These studies support Brenner’s hypothesis that individuals with nephron deficit are more likely to develop hypertension and renal disease, following compensatory glomerular hypertrophy. Therefore, identifying factors that result in congenital nephron deficit will allow better understanding of how accelerated risk for such conditions is established.

1.4 PAX2

PAX2 is one of the nine members of the paired-box (PAX) gene family of transcription factors important for the development of various organs and tissues (Stayner, Cunliffe, Ward, & Eccles, 1998). All nine members of PAX genes family encode protein products that contain a highly conserved 128-amino acid DNA-binding motif, referred to as paired-box domain, in the
N-terminal region of the molecule. (Dahl, Koseki, & Balling, 1997; Stuart, Kioussi, & Gruss, 1994). *PAX2*, located on chromosome 10 in humans and on chromosome 19 in mice, plays an essential role in regulating the development of the optic nerve, ears, central nervous and urogenital systems (Dahl et al., 1997; Dressler, Deutsch, Chowdhury, Nornes, & Gruss, 1990; Eccles et al., 1992; Nornes, Dressler, Knapik, Deutsch, & Gruss, 1990). *Pax2* knockout mice and mice with heterozygous mutation in the *Pax2* gene exhibit malformations of the urogenital tract, the optic nerve and the central nervous system (Favor et al., 1996; Torres, Gomez-Pardo, Dressler, & Gruss, 1995). In humans, *PAX2* mutations have been observed in patients with renal hypoplasia and defects in the optic nerves. Therefore, tight regulation of the *PAX2* gene is crucial for proper renal development.

### 1.4.1 *PAX2* in Kidney Development

Among the many regulators that are involved in the complex process of kidney development, *PAX2* is one of the earliest transcription factors expressed in embryonic kidney and remains a key regulator throughout urogenital system development. During kidney development, the highest levels of *PAX2* expression in WD and the branching UB and a rapid downregulation of *PAX2* expression is observed once nephrogenesis is complete, emphasizing the role of *PAX2* in the early patterning of the metanephros (Dressler et al., 1990; Lechner & Dressler, 1997; Torres et al., 1995). It has been suggested that *PAX2* plays multiple roles during kidney development. It is involved in the activation of glial cell-derived neurotrophic factor (GDNF) in the MM, an important factor that initiates the invasion of the MM by the UB (Brophy, Ostrom, Lang, & Dressler, 2001). *PAX2* also plays an important role in inducing mesenchymal-to-epithelial transition (MET), which accompanies nephrogenesis (Rothenpieler & Dressler, 1993; Torban et al., 2000). Recently, the role of *PAX2* as an anti-apoptotic gene in the UB during
kidney development has been investigated. Elevated apoptotic UB cells and reduced nephron numbers were observed in fetal kidneys of mice with heterozygous mutation in the Pax2 gene (Pax21Neu) (Porteous et al., 2000) and PAX2 was shown to suppress apoptotic cell death in cultured renal cells (Torban et al., 2000).

Eccles et al. proposed a model to explain the anti-apoptotic effect of PAX2 on branching morphogenesis (Figure 1.1) (Eccles et al., 2002). According to this model, an inhibitory field is formed by the condensing mesenchymal cells surrounding the growing UB. In order for the next generation of nephrons to be produced, the UB will need to grow beyond this inhibitory field. PAX2 may function at the interface of proliferation and differentiation to protect UB from apoptosis, which may explain why PAX2 haploinsufficiency results in reduced UB branching and consequently renal hypoplasia as observed in humans and mice with PAX2 mutations. To further investigate whether apoptosis in the growing UB alone can cause renal hypoplasia, Dziarmaga et al. targeted an apoptosis-inducing transgene to Pax2-expressing fetal kidney cells and observed increased apoptosis and reduced nephron number caused by reduced UB branching (Dziarmaga et al., 2003). Another study demonstrated that branching defects caused by PAX2 mutations can be rescued by suppressing UB cell apoptosis with caspase inhibitor (Clark et al., 2004), which further supports the PAX2 apoptosis model postulating the anti-apoptotic role of PAX2 in UB branching.
1.4.2 PAX2 and Renal Hypoplasia

The genetic influences that determine the final nephron number during nephron maturation remains unknown. Renal-coloboma syndrome (RCS) is a rare autosomal dominant condition in which the structure of the nephron does not appear to be greatly affected, but a huge decrease in nephron number is observed (Salomon et al., 2001). RCS can be characterized by renal hypoplasia, a primary feature of the syndrome, accompanied by ocular colobomas, vesico-ureteral reflux, brain malformation or ear defects (Sanyanusin et al., 1995). RCS is caused by heterozygous null mutations of the PAX2 gene, a key developmental gene (Eccles & Schimmenti, 1999; Porteous et al., 2000; Sanyanusin et al., 1995). In fact, at least 10 different mutations in the human PAX2 gene was reported to be associated with RCS. Mutations in PAX2 are also observed in children who were diagnosed with oligomeganephronia, a condition that exhibits renal hypoplasia with no apparent ocular defects (Salomon et al., 2001). In both conditions, aside from the compensatory glomerular hypertrophy, the renal morphology seems unaffected and therefore, renal insufficiency must be caused by nephron deficit. It seems clear then that PAX2 is involved.
in the regulation of parts of the kidney development associated with determining the final nephron endowment.

1.4.3 Polymorphisms in the PAX2 gene

There is a growing interest in determining whether common polymorphisms in the PAX2 gene may be associated with subtle reduction in nephron number in normal newborns born with nephron numbers at the lower end of the spectrum, who do not display any signs of conditions that may cause renal hypoplasia. Our lab has previously demonstrated an association between subtle renal hypoplasia and common haplotype-tagging single-nucleotide polymorphisms (htSNP) in the PAX2 gene, identified using the HapMap project data (Quinlan et al., 2007). Five htSNP variants were reported to be associated with a significant reduction in kidney volume, a surrogate for nephron number (Figure 1.2). Four of these htSNP were found in a region that spanned intron 3 to intron 5 of the PAX2 gene on chromosome 10. Out of the five htSNP variants, three of them (rs11599825, rs11190688, rs11190702) were relatively common and in tight linkage disequilibrium (LD) with each other. These htSNP were used to construct PAX2 haplotypes GGG or AAA, which were assigned to individuals in the cohort. 28% of the cohort population was heterozygous for the AAA haplotype and 5% of the cohort population was homozygous for the allele (AAA/AAA), and having at least one PAX2 AAA haplotype conferred the individuals with kidney volume that was reduced by 10% compared to individuals with the more common GGG/GGG haplotype. The other htSNP found in the PAX2 gene (rs1800898) was used for allelic imbalance studies, which showed a reduced PAX2 mRNA expression in the less common rs1800898(C) allele compared to the more common rs1800898(A) allele. These results provide evidence that a common variant of the PAX2 gene is associated with subtle renal hypoplasia in
newborns, which may contribute to the risk for essential hypertension or susceptibility to renal damage in adulthood.

Figure 1.2 Linkage disequilibrium (LD) between haplotype-tagging single-nucleotide polymorphisms (htSNP) in the PAX2 gene associated with renal hypoplasia. The PAX2 region on chromosome 10 with twenty-three htSNP that span the region is shown. The value inside each square indicates the extent of (D') of LD between htSNP. htSNP highlighted in gray were shown to be associated with reduced kidney volume and three of the five htSNP that were used to construct haplotypes are indicated. The SNP used for allelic imbalance studies in exon 8 is indicated in green.

1.4.4 Pax2 Mouse Models

Much of our understanding of molecular regulation of kidney development was made possible by the analysis of genetically altered or knock-out mice. Thus far, three mouse models
have been developed for the purpose of studying the role of PAX2 in kidney development: the 
Krd mouse, which contains a deletion in parts of the Pax2 gene (S. A. Keller et al., 1994), Pax2 
knockout mouse(Torres et al., 1995) and Pax21Neu mouse (Favor et al., 1996) with the last two 
models containing mutations that affect the paired-box region in the Pax2 protein. In all three 
mouse models, heterozygous mutations were associated with renal hypoplasia whereas 
homezygous inactivation of Pax2 gene was lethal and caused bilateral renal agenesis. Pax21Neu 
mouse has been the most popular model for studying the role of Pax2 in renal hypoplasia 
because it carries a 1-bp insertion, which resembles the mutation observed in the humans with 
RCS.

Pax21Neu mouse is characterized by a frameshift mutation caused by a spontaneous single 
insertion of guanine in exon 2 of the Pax2 gene which results in a premature stop codon and 
ence non-functional protein (Favor et al., 1996). Heterozygous Pax21Neu mice are born with 
renal hypoplasia associated with ocular defects and homezygous Pax21Neu mice embryos 
exhibited malformations in the mid-hindbrain region, the optic nerve and ventral regions of the 
inner ear. Studies using the Pax21Neu mouse strain demonstrated a decrease in congenital 
nephrone number by 40%, reduction in renal cross-sectional area and glomerular hypertrophy 
pared to the wildtype littermates (Dziarmaga et al., 2006). Taken together, Pax21Neu mouse 
will serve as a good model for studying renal hypoplasia and its risk factor for hypertension and 
renal injury.

1.5 Diabetes Mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia that 
results from defects in insulin production or decreased insulin sensitivity (Alberti & Zimmet, 
1998; Dabla, 2010). It is an extremely prevalent disease, affecting more than 387 million adults
worldwide and is expected to be on the rise and is expected to affect more than 592 million adults by the end of the year 2030 (Bhatti & Usman, 2015). What makes diabetes such a serious and life-threatening disease is the numerous complications it can lead to, increasing the risk of various diseases. Diabetes is associated with many long-term complications such as diabetic nephropathy, peripheral diabetic neuropathy, diabetic retinopathy and cardiovascular complications. Among them, diabetic nephropathy (DN) is considered the most prevalent and severe complication, affecting as much as one-third of diabetics (Reutens & Atkins, 2011). In fact, diabetes is the major cause of end-stage renal disease (ESRD) globally, accounting for 30-47% cases of ESRD. The health and financial costs of DN are at their highest yet, constituting a tremendous socioeconomic burden on the society (Xue et al., 2017). Therefore, there is an urgent need to prevent DN and its progression in order to reduce the morbidity and mortality associated with ESRD.

1.5.1 Pathophysiological Process of Diabetic Nephropathy

Mechanisms that lead up to the development and progression of DN remain unclear. However, it is generally agreed that hyperglycemia is the key trigger and the sustaining factor that causes alterations in renal function and structure throughout the progression of DN. In fact, glucotoxicity is a high risk factor for the development of microvascular complications, which lead to tissue and organ damages. The first characteristic feature of DN is the thickening of the glomerular basement membrane (GBM), followed by the thickening of the tubular basement membrane (TBM) and increased deposition of mesangial matrix, compromising the glomerular surface area that is available for filtration (Brito et al., 1998; Goode, Shires, Crellin, Aparicio, & Davison, 1995). Damage to podocytes is an important indicator of renal dysfunction in DN. Podocytes are terminally differentiated epithelial cells that wrap around the capillaries of
the glomerulus and play a crucial role in selectively filtering molecules in the blood into the urine as the glomerular filtration barrier (Barisoni & Mundel, 2003; Greka & Mundel, 2012). Disruption of this filtration barrier allows larger proteins to leak into the tubular fluid and results in proteinuria. Hyperglycemia is also commonly associated with hypertension, another major risk factor for DN that puts a burden on renal blood vessels and contributes to the impairment of the glomerulus. Hypertension associated with DN is often characterized by fluid retention and increased peripheral vascular resistance caused by damage in the arteries (Epstein & Sowers, 1992). Altogether, these renal alterations cause glomerular and tubular sclerosis, which in turn further decreases the filtration surface area and triggers a vicious cycle that eventually leads to renal failure.

1.5.2 Biomarkers of Diabetic Nephropathy

Albuminuria is one of the earliest and most important biomarkers to diagnose DN. Albumin is the most common protein found in the urine and is normally excreted in very small amounts in healthy individuals. Microalbuminuria is detected in the initial stages of DN with albumin excretion of 30-300 mg in a 24h urine collection and progresses to an extensive proteinuria with albumin excretion of >300 mg/24h as the kidney becomes severely damaged in the later stages of the disease (American Diabetes, 2005; Tabaei, Al-Kassab, Ilag, Zawacki, & Herman, 2001). Recently, measuring urine albumin-to-creatinine ratio (ACR) has become the preferred method for detecting early signs of DN. Creatinine is a chemical waste product produced by muscle metabolism and is normally filtered out from the blood and excreted in the urine. As such, ACR is predicted to be a more accurate measurement of protein excretion in the urine because it corrects for the variations in urine concentrations due to hydration. In humans, ACR measurement of 30-300 µg/mg is considered microalbuminuria and ACR greater than 300
µg/mg can be detected in proteinuria (American Diabetes, 2005). In some cases, decreased GFR is observed in patients with normal urinary albumin excretion (UAE) rate, making estimation of GFR essential in the assessment of renal function (Caramori, Fioretto, & Mauer, 2003).

GFR measures the rate at which the glomeruli filter plasma for removal of waste products from it and is considered the best parameter of kidney function since it accounts for age, BMI and sex. GFR is generally estimated by measuring the renal clearance of an endogenous marker in plasma that is solely filtered by the glomerulus, expressed as the volume of plasma cleared of the marker per unit time. GFR in healthy adults is 90-120 ml/min/1.73 m² (Levey et al., 2005). Serum creatinine is the most commonly used marker for estimating GFR because plasma creatinine concentration is very consistent and once it is filtered from the blood into the urine, almost none of it is reabsorbed. However, recent studies have demonstrated that serum cystatin C may be a better predictor of kidney function and a more sensitive indicator of mild renal impairment compared to serum creatinine (Dharnidharka, Kwon, & Stevens, 2002; Roos, Doust, Tett, & Kirkpatrick, 2007; Sarnak et al., 2005).

1.5.3 Progression of Diabetic Nephropathy

DN in type I diabetes mellitus progresses in series of stages with each stage characterized by specific alternations in renal structure and function. Stage 1 and 2, defined as pre-diabetic nephropathy, can be characterized by hyperfunction, hypertrophy and a surge in the glomerular filtration rate (GFR) (Mogensen, Christensen, & Vittinghus, 1983). These renal changes represent the compensatory short-term strategy for the kidney to maintain its function while long-term regenerative processes take place. However, these renal changes are the contributing risk factors for subsequent renal damage and the development of DN. In stage 3, referred to as incipient DN, a slow, gradual increase in urinary albumin excretion is observed along with an
increase in blood pressure. Microalbuminuria level is slightly above the normal range but does not reach the values observed in clinical disease. GFR remains close to the normal level at this stage but begins to decline in overt DN, stage 4 of the disease progression. Stage 4 is characterized by high amount of urinary albumin excretion and associated hypertension. With persistent proteinuria, progression to ESRD, stage 5 of the disease, is inevitable. It is therefore imperative to recognize the onset of renal damage to plan further management to reverse the progression of this disease. At present, microalbuminuria is the most reliable feature to predict early renal damage in diabetic patients. However, in a small subset of diabetic individuals, DN develops earlier and progresses at a faster rate than others, pointing out the need for the development of alternative methods to distinguish and identify these patients.

Figure 1.3. Progression of diabetic nephropathy in stages.
(Rossing, Peter. Clinical pathology of nephropathy [internet]. 2015 Sep 23; Diapedia 71040851172 rev. no. 10. Available from: https://doi.org/10.14496/dia.71040851172.10)

1.5.4 Fast Renal Decliners in Diabetic Nephropathy

The complex interplay between genetic and environmental factors is the determinant of an individual’s likelihood of developing DN. There is wide variation in the rate at which diabetic
nephropathy progresses, independent of diabetic control (Krolewski, Skupien, Rossing, & Warram, 2017). Susceptibility to nephropathy shows family clustering in siblings with diabetes. However, mapping out genes that increase susceptibility to nephropathy is difficult due to the genetic heterogeneity in the human population and the variability in environmental factors, such as treatment methods. Recently, the influence of genetic variation has been confirmed by recent Genome-Wide Association Studies (GWAS) but it has only led to the identification of loci with marginal clinical effects (Tanaka & Babazono, 2005; Teumer et al., 2016). Discovering factors that predispose some individuals to accelerated progression of DN has been receiving great amount of attention as potential diagnostic and treatment measures. This brings us back to Brenner’s hypothesis, which states that reduction in congenital nephron number may account for the susceptibility of diabetics to develop renal disease (Brenner et al., 1988). If decrease in nephron number indeed causes accelerated renal deterioration in diabetics, we will be able to detect fast renal decliners amongst diabetic patients, which could result in the development of effective diagnosis and treatment of DN.

1.5.5 Diabetes and Mouse Models

While current mouse models of diabetes are not fully representative of functional and pathological characteristics of human DN, they are nonetheless useful tools for studying the pathogenesis and treatment of kidney disease. A mouse model of diabetes was discovered in a colony of C57BL/6 mice in Akita, Japan (Yoshioka, Kayo, Ikeda, & Koizumi, 1997) and since the discovery, the Akita mouse has been used as a promising genetic model of type I diabetes. There is a misfolding of the insulin protein in the Akita mice which carries an autosomal dominant, spontaneous point mutation that results in an amino acid substitution in the A chain of mature insulin (\textit{Ins}2\textsuperscript{C96Y}). The proteotoxicity of the misfolded proteins cause a depletion of \(\beta-\)
cells in the islets of Akita mice and as a result, insufficient amount of mature insulin is released (Ellgaard, Molinari, & Helenius, 1999; Wang et al., 1999). Akita mice carrying homozygous mutation are perinatally lethal while mice carrying heterozygous mutation develop hyperglycemia at around 3-4 weeks of age. To date, several Akita mouse models have been generated on different inbred genetic background, each with different severity of DN.

Streptozotocin (STZ) is also a commonly used method in diabetic research. STZ is a compound that has a cytotoxic effect on the pancreatic islet β cells (Lenzen, 2008). Multiple low doses of STZ have been shown to induce moderate hyperglycemia and pancreatic damage similar to that observed in type I diabetes in animals. However, there is a controversy in the use of STZ to initiate diabetes in animals. High doses of STZ have been associated with massive necrosis of the pancreatic β-cell mass and potential toxicity to a number of other tissues, which may have a confounding effect on the interpretation of results (Breyer et al., 2005). In addition, a study which compared the effect on kidney injury in the Akita mice and STZ-treated mice of the same strain demonstrated that the renal pathological features associated with diabetes was more profound in the Akita mice relative to STZ-treated mice (Gurley et al., 2006). Therefore, genetic models of type I diabetes may offer advantages over chemical models of diabetes, making Akita model an ideal model for studying the mechanisms underlying the pathogenesis of DN.

1.6 Thesis Proposal and Research Aims

Although kidney development is a process that involves a complex interaction between various regulators, our lab has demonstrated through various studies that $PAX2$ is an essential regulator of UB branching and nephrogenesis (Clark et al., 2004; Porteous et al., 2000; Quinlan et al., 2007; Torban et al., 2000). It has long been recognized that there is a wide variation in the number of nephrons between individuals in humans. Investigation of the association between
nephron number and susceptibility to the development of hypertension and renal diseases has been receiving a lot of interest. The idea was first proposed by Brenner et al., stating that individuals born with suboptimal nephron numbers are at higher risk for the development of hypertension and acquired renal diseases (Brenner et al., 1988).

Based on Brenner’s early observations and our discovery that PAX2 is a major regulator of nephron number, we hypothesize that diabetic patients with dysfunctional PAX2 allele exhibit accelerated nephropathy. The first aim of my project is to determine whether heterozygous null Pax2 allele accelerates the progression of diabetic nephropathy in vivo using a mouse strain generated by crossing Pax2 mutant mice with diabetic mice. Our second approach was aimed to understand the early course of diabetic nephropathy in humans by analyzing clinical data from a cohort of young diabetic patients and performing genome-wide analysis of this cohort using SNPs to test for an association between a common haplotype of the PAX2 gene and early onset of diabetic nephropathy.
CHAPTER II: Heterozygous Pax2 mutation accelerates the progression of DN and induces hypertension in diabetic mice
2.1 Overview

Nephrogenesis in mammals is induced early in fetal life when the ureteric bud (UB) arises from the Wolffian duct, grows outward into the metanephric mesenchyme (MM) and begins branching (Dziarmaga et al., 2006; Little et al., 2007). Branching morphogenesis is an important determinant of an individual’s final nephron endowment. In humans, nephron numbers vary greatly between individuals ranging from 210,000 to 2,700,000 (Bertram et al., 2011). Brenner et al. were the first to suggest a clinical importance of nephron numbers and hypothesized that congenital nephron deficit may be associated with susceptibility to acquired renal disease and essential hypertension in adulthood (Brenner et al., 1988).

*PAX2* is a transcription factor that plays an essential role in kidney development. *PAX2* is expressed in the emerging UB and subsequently at the tip of induced MM and the epithelial structures of the emerging nephron (Dressler et al., 1990; Eccles et al., 1992; Torres et al., 1995). The importance of *PAX2* in nephrogenesis has been demonstrated in mice with a heterozygous mutation in the *Pax2* gene (*Pax2<sup>1Neu</sup>), which exhibits kidney morphology identical to that observed in human renal-coloboma syndrome (RCS) (Favor et al., 1996; Torban et al., 2000). While several roles of *PAX2* in kidney development have been suggested, its anti-apoptotic function in the arborizing UB to promote its growth and branching has been implicated in recent studies (Clark et al., 2004; Dziarmaga et al., 2003; Dziarmaga et al., 2006; Porteous et al., 2000; Torban et al., 2000), defining *PAX2* as a central player in determining nephron endowment.

Since mutation in the *PAX2* gene is associated with renal hypoplasia, we hypothesized that *Pax2* mutation in mice, with suboptimal nephron number, will be more susceptible to nephropathy and essential hypertension in the setting of diabetes. To test the hypothesis, we crossed mice with heterozygous mutation in *Pax2* gene (*Pax2<sup>1Neu</sup>*) with diabetic mice with
heterozygous mutation in Ins2 gene (Ins2<sup>4kiba</sup>). Using this crossed mouse model, we examined the progression of diabetic nephropathy (DN) by measuring the blood pressure, urinary albumin-to-creatinine ratio (ACR) and serum cystatin C. We also measured the glomerular size to check for compensatory hypertrophy and filtration in mice born with reduced nephron numbers. Nephron numbers in these mice were counted to confirm the effect of Pax2 mutation on nephrogenesis. To validate that the worse pathological features observed in diabetic mice with Pax2 mutation is in fact due to reduced nephron number, we performed a rescue experiment using a caspase inhibitor to suppress UB cell apoptosis.

2.2 Materials and Methods

2.2.1 Mouse Breeding

All wildtype and 1Neu mouse colonies of CD1 background were bred and maintained at the McGill University Health Centre Research Institute (MUHC-RI) vivarium in Montreal, Canada. All wildtype and Ins2 mouse colonies of C57BL/6 background were purchased from the Jackson Laboratory. Breeding was established by crossing Pax2<sup>1Neu</sup> mice of CD1 background with Ins2<sup>4kiba</sup> mice of C57BL/6 background. Litters born to these mice will be of strain derived from CD1 and C57BL/6 (CD1;B6) and will have the following genotypes: Pax2<sup>+/+</sup>;Ins2<sup>+/+</sup>, Pax2<sup>+/+</sup>;Ins2<sup>-/-</sup>, Pax2<sup>-/-;Ins2<sup>+/+</sup></sup>, Pax2<sup>-/-;Ins2<sup>-/-</sup></sup>. All mice were housed in MUHC-RI vivarium and all animal studies were performed in agreement with the regulations of the Canadian Council on Animal Care (CCAC). Animal protocols were approved by the McGill University Animal Care Committee (UACC, AUP #5767).

2.2.2 Genotyping

All genotyping was completed using DNA extracted from tail tissue samples from mice.
2.2.2.1 Pax2\(^{1Neu}\)

Genotyping method for \(1Neu\) mice involved a combination of polymerase chain reaction (PCR) and restriction enzyme digest. A restriction site for XcmI was created using specially designed primers which induce a guanine (G) to thymine (T) substitution in the \(1Neu\) mutant but not the wild-type sequence (Figure 2.1). The forward primer used for PCR is 5’ GTGTGAACCAGCCGGGGGTG 3’ and the reverse primer is 5’ GCCCAGGATTTTGCTGACACAGCC 3’. The PCR-amplified products were digested with XcmI (New England Biolabs, MA, USA). The digested samples were run on 10% polyacrylamide gels which were subsequently visualized using ethidium bromide staining. \(Pax2^{+/+}\) displayed a single band at 166 bp and heterozygous \(1Neu\) (\(Pax2^{+/-}\)) mice displayed two bands at 166 bp and 151 bp.

![Diagram showing genotyping of Pax2\(^{1Neu}\) mice](image)

**Figure 2.1 Genotyping \(Pax2^{1Neu}\) mice.** (Clark et al., 2004)
2.2.2.2 $\text{Ins}2^{\text{Akita}}$

Genotyping for Akita mice was performed using a combination of PCR and restriction enzyme digest. In the $\text{Ins}2$ mutant allele, guanine (G) is substituted with adenine (A) at nucleotide 1907, disrupting Fnu4HI site. The forward primer used for PCR is 5’ TGCTGATGCCCCTGGCCTGCT 3’ and the reverse primer is 5’ TGGTCCCACATATGCACATG 3’. The PCR products were digested with Fnu4HI (New England Biolabs, MA, USA). The digested samples were run on 10% polyacrylamide gels and visualized by ethidium bromide staining. $\text{Ins}2^{+/+}$ showed one band at 140 bp and heterozygous $\text{Ins}2$ ($\text{Ins}2^{+/-}$) showed two bands at 140 bp and 280 bp (Figure 2.2).

![Figure 2.2 Genotyping $\text{Ins}2^{\text{Akita}}$ mice. (Lu, Sternini, Rozengurt, & Zhukova, 2005)](image-url)
2.2.3 Blood Pressure Measurements

Systolic blood pressure measurements were taken biweekly in conscious, restrained mice using the BP-2000 Blood Pressure Analysis System™ (Visitech Systems Inc., NC, USA). The BP-2000 is a computerized, non-invasive tail-cuff system with a tail cuff inflator and a phptoelectric sensor. Measurements were taken in mice aged 13 weeks old until 19 weeks old. Due to the controversy on the accuracy of the diastolic blood pressure measured using the BP-2000 system, only systolic blood pressure measurements were recorded. Blood pressure measurements were performed 2-3 times a week for 3 weeks before the actual measurements were recorded to acclimatize the mice to the machine. Significance of the difference in means was determined by the paired \( t \) test.

2.2.4 Measurement of Urinary Albumin Excretion (UAE)

Excretion of urinary albumin was determined using albumin-to-creatinine ratio (ACR) on single time point spot urine collected by stimulation of live male and female mice or by direct withdrawal from the bladder after sacrifice using a syringe. Spot urine was collected every other week from week 14 of age until week 20, when the mice were sacrificed. All urine collections were performed at the same time of the day. To ensure a proper separation of liquid urine and other remaining solid components, urine samples were centrifuged at 13,000 x g for 10 min at 4°C. The supernatant was snap frozen and stored at -80°C. The concentration of albumin in the urine was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA). Creatinine in the urine samples was quantified using enzymatic Mouse Creatinine Assay Kit (Crystal Chem, IL, USA). The experiments were performed according to the manufacturers’ protocols. The concentrations of albumin and creatinine in the urine samples were used to
calculate ACR and represented as mean ± SEM. Significance of the difference in means was determined by two-way ANOVA.

### 2.2.5 Measurement of glomerular filtration rate (GFR)

Renal function in male and female mice was evaluated by measuring the cystatin C from serum samples. Blood was collected by cardiac puncture immediately after sacrifice. Blood samples were collected in Microvette® 500 Z-Gel tubes (Sarstedt, Nümbrecht, Germany). Serum samples were separated by centrifugation at 3,000 x g for 10 min, snap frozen and stored at -80°C. The concentration of cystatin C in serum samples were determined using Mouse/Rat Cystatin C Quantikine ELISA Kit (R&D Systems, MN, USA). Measurements were made according the manufacturer’s instructions and represented as mean ± SEM. Significance of the difference in means was determined by unpaired t test.

### 2.2.6 Nephron Counting

Litters from *Pax2*<sup>1Neu</sup> and *Ins2*<sup>Akita</sup> crosses were sacrificed at week 20 and their final nephron numbers were estimated. Both left and right kidneys were removed from mice at time of sacrifice and fixed in 4% paraformaldehyde (PFA) overnight. The kidneys were dehydrated in 70% ethanol. The left kidneys were embedded in paraffin and the right kidneys were stored in 70% ethanol in 4°C. Serial sagittal sections of the embedded kidneys were made at 7 µm in thickness and every 10<sup>th</sup> section was mounted on slides. Slides were stained with haemotoxylin and eosin (H&E) and photographed at 10X objective using Canon Powershot S50 attached to Axiophot microscope (ZEISS, Oberkochen, Germany). Images were taken using AxioVision 4.8.2 Service Pack 2 software. Images were analyzed using Image J software and individual glomerulus was counted using the multi-point tool. Total nephron number in the whole left kidney was estimated using a formula developed by Murawski et al. (Murawski et al., 2010): \[ N_{\text{glomeruli}} = f \times 0.4 \times NN, \]
where \( f \) is the fraction of the sections used from the whole kidney, \( NN \) is the total number of nephrons counted and 0.4 is a constant which corrects for overlapping glomeruli when all glomeruli on one section are counted. In order to eliminate any factors that can lead to variations in results, only male kidney samples were used. Nephron numbers are represented as mean ± SEM and the significance of the difference was tested by unpaired t test.

2.2.7 Measurement of Glomerular Size

The same H&E stained left kidney sections from male mice prepared for nephron counting were used to evaluate hypertrophy expected in \( \text{Pax2}^{+/+} \) and \( \text{Ins2}^{+/+} \) mice. From all of the 7 µm sections mounted on slides, five evenly-spaced sections were selected per kidney for glomerular size measurement. Kidney sections were imaged at 10X objective using Canon Powershot S50 attached to Axiophot microscope (ZEISS, Oberkochen, Germany). Using the oval selection tool from Image J software, the area of each glomerulus was measured and converted from pixels to µm after calibrating for image magnification. The largest 20 glomeruli in each of the five sagittal sections were used to calculate the mean glomerular area in each kidney sample.

2.2.8 Administration of Pro-VAD-FMK

In order to rescue the defective nephrogenesis in mice carrying \( \text{Pax2} \) mutation, we administered a pan caspase inhibitor, Pro-VAD-FMK during renal development. Pro-VAD-FMK (Vergent Bioscience, MN, USA) is the fastest and the most potent caspase inhibitor on the market. \( \text{Pax2}^{1\text{Neu}} \) and \( \text{Ins2}^{\text{Akita}} \) mice were left overnight for mating and the presence of vaginal plug was recorded as embryonic day E0.5. Impregnated females received daily intraperitoneal (IP) injections of Pro-VAD-FMK (5 µg/g body weight) from E13.5 to E20.5. Litters will be
assessed for blood pressure, urinary ACR, serum cystatin C, nephron number and glomerular size using the same methods mentioned above.

2.3 Results
2.3.1 Heterozygous Pax2 mutation alone (Pax\(^{+/−}\);Ins2\(^{+/−}\)) does not exhibit early onset of renal impairment

We wanted to evaluate whether reduced nephron number caused by mutation in Pax2 is sufficient to accelerate the development of hypertension and the progression of nephropathy in our crossed background (CD1;B6). Therefore, we first compared Pax\(^{+/−}\);Ins2\(^{+/−}\) mice born from Pax2\(^{1Neu}\) and Ins2\(^{Akita}\) crosses with their littermates with no mutations (Pax\(^{+/+}\);Ins2\(^{+/+}\)) and assessed for any signs of early renal damage and hypertension.

2.3.1.1 Heterozygous Pax2 mutation causes congenital nephron deficit

Heterozygous Pax2 mutation has been shown to cause nephron deficit in mice. The effect of heterozygous Pax2 mutation alone in the cross strain of CD1;B6 background on nephrogenesis was confirmed by estimating the total nephron number in Pax\(^{+/−}\);Ins2\(^{+/−}\) mice. Average nephron number calculated from three kidney samples was 22993 in Pax\(^{+/−}\);Ins2\(^{+/−}\) mice, which was 40.3% less than the average nephron number in their littermates with no mutations (Pax\(^{+/+}\);Ins2\(^{+/+}\)), which was 38536 (Figure 2.3). The difference was significant with p-value of 0.0425.
Figure 2.3 Nephron number in $Pax^{+/+};Ins2^{+/+}$ vs $Pax^{+/-};Ins2^{+/+}$ at week 20. Total nephron number in the left kidneys is significantly reduced in mice with heterozygous $Pax2$ mutation ($Pax^{+/-};Ins2^{+/+}$: 22993 ± 3657, n = 3) compared to their littermates with no mutations ($Pax^{+/+};Ins2^{+/+}$: 38544 ±5765, n = 3), $p = 0.0425$.

2.3.1.2 Heterozygous Pax2 mutation causes moderate glomerular hypertrophy

Left kidneys collected at the age of week 20 were analyzed for signs of early glomerular hypertrophy in $Pax^{+/-};Ins2^{+/+}$ mice. 20 largest glomeruli were recorded from five evenly-spaced sections in each kidney sample (100 glomeruli per kidney). Average glomerular area in three kidney samples from $Pax^{+/-};Ins2^{+/+}$ mice (glomerular area = 5966.59 µm$^2$) showed a significant, moderate increase of 20.6% compared to average glomerular area in $Pax^{+/+};Ins2^{+/+}$ mice (glomerular area = 4946.05 µm$^2$ n=3) (Figure 2.4).
Heterozygous Pax2 mutation causes glomerular hypertrophy in the left kidney of mice at week 20. Glomerular area are shown as mean ± SEM: $Pax^{+/+};Ins2^{+/+}$ (4946 ± 279.8 µm$^2$, n = 3); $Pax^{+/-};Ins2^{+/+}$ (5967 ± 317.8 µm$^2$, n = 3), $p = 0.0368$.

2.3.1.3 Heterozygous Pax2 mutation has no effect on blood pressure

Bi-weekly systolic blood pressures were recorded in litters from $Pax2^{Neu}$ and $Ins2^{Akita}$ between week 13 and 19. The mean blood pressures for each week in $Pax^{+/-};Ins2^{+/+}$ mice (n = 17) were compared to those for $Pax^{+/+};Ins2^{+/+}$ mice (n = 23) to assess whether Pax2 mutation results in early onset of hypertension. No significant difference was observed in the systolic blood pressure between $Pax^{+/-};Ins2^{+/+}$ mice and $Pax^{+/+};Ins2^{+/+}$ littermates during these time points ($p = 0.21$) (Figure 2.5).
2.3.1.4 Heterozygous Pax2 mutation does not show signs of renal damage by week 20

Urine samples were collected in litters from Pax2<sup>1Neo</sup> and Ins2<sup>Akita</sup> at weeks 14, 16, 18 and 20. To evaluate whether congenital nephron deficit induces renal damage at these early time points, we compared urinary albumin-to-creatinine ratio (ACR) between Pax<sup>+/−;Ins2<sup>+/+</sup></sup> and Pax<sup>+/++;Ins2<sup>+/+</sup></sup> littermates and observed no significant differences between the two genotypes (Figure 2.6). The results indicate that nephron deficit does not lead to albuminuria in the early adulthood in mice.

To further test for signs of nephropathy in mice with congenital deficit, we tested for early signs of reduced GFR using serum cystatin C concentrations measured from blood samples collected in Pax<sup>+/−;Ins2<sup>+/+</sup></sup> at week 20. Mean serum cystatin C concentration in Pax<sup>+/−;Ins2<sup>+/+</sup></sup> mice (n = 15) was 326.0 ± 17.35 ng/mL, which was almost identical to that of Pax<sup>+/++;Ins2<sup>+/+</sup></sup>
mice (322.1 ± 16.24 ng/mL, n = 20), indicating that *Pax2* mutation alone does not affect the GFR in early adulthood (Figure 2.7).

**Figure 2.6** Albumin-to-creatinine ratio (ACR) in *Pax2*+/+;*Ins2*+/+ vs *Pax2*+/-;*Ins2*+/+ at weeks 14, 16, 18 and 20. Urinary albumin excretion (UAE) of *Pax2*+/-;*Ins2*+/+ (n = 17) did not show any significant difference from UAE of *Pax2*+/+;*Ins2*+/+ (n = 18) and remained at steady levels at all time points between 14 to 20 weeks.
Figure 2.7 Serum cystatin C concentrations in $Pax^{+/+};Ins2^{+/+}$ vs $Pax^{+/+};Ins2^{+/+}$ suggests no difference in GFR at week 20. At week 20, the serum cystatin C level was observed to be unaffected by reduced nephron number. Mean serum cystatin C concentrations were not significantly different between $Pax^{+/+};Ins2^{+/+}$ and $Pax^{+/+};Ins2^{+/+}$ mice.

2.3.2 Heterozygous Ins2 mutation alone ($Pax^{+/+};Ins2^{+/-}$) exhibits marginal signs of early DN

To determine how early diabetes progresses towards DN in our crossed strain of CD1;B6, we compared diabetic $Pax^{+/+};Ins2^{+/-}$ mice with no defects in nephrogenesis with their littermates with no mutations ($Pax^{+/+};Ins2^{+/+}$). Features of renal impairment were evaluated for signs of DN.

2.3.2.1 Total nephron number in $Pax^{+/+};Ins2^{+/-}$

To confirm that nephron deficit is solely caused by Pax2 mutation, we counted nephrons of diabetic mice in the absence of Pax2 mutation ($Pax^{+/+};Ins2^{+/-}$) to assess whether they were comparable to their littermates that lack both Pax2 and Ins2 mutations ($Pax^{+/+};Ins2^{+/+}$). Average nephron number for three mice of genotype $Pax^{+/+};Ins2^{+/-}$ was $41613 \pm 2903$ (n = 3), which was not significantly different from the average nephron number of $Pax^{+/+};Ins2^{+/+}$ littermates as expected ($38544 \pm 5765$, n = 3) (Figure 2.8).
Figure 2.8 Total nephron number is unaffected in the absence of Pax2 mutation. Total nephron numbers from $Pax^{+/+};Ins2^{+/+}$ (41613 ± 2903, n = 3) and $Pax^{+/+};Ins2^{+/+}$ (38544 ± 5765, n = 3) male mice at week 20 were not significantly different.

2.3.2.2 Diabetes causes glomerular hypertrophy by week 20 in $Pax^{+/+};Ins2^{+/+}$ mice

Diabetes is associated with early renal hypertrophy that precedes irreversible renal changes of ESRD such as glomerulosclerosis (Ziyadeh, 1993). To investigate whether diabetes contributes to glomerular hypertrophy in litters from $Pax^{2lNeu}$ and $Ins2^{Akita}$ by week 20, mean glomerular areas of $Pax^{+/+};Ins2^{+/+}$ and $Pax^{+/+};Ins2^{+/+}$ litters. By week 20, $Pax^{+/+};Ins2^{+/+}$ mice (n = 3) exhibited signs of glomerular hypertrophy with mean glomerular area of 6586 ± 562.3 µm², which was 33.2% greater than mean glomerular area of $Pax^{+/+};Ins2^{+/+}$ mice (4946 ± 279.8 µm², n = 3), p-value = 0.0297 (Figure 2.9).
Glomerular hypertrophy is observed in diabetic \textit{Pax}^{+/+};\textit{Ins2}^{+/−} mice at week 20. Presence of diabetes results in increased glomerular area in \textit{Pax}^{+/+};\textit{Ins2}^{+/−} mice in comparison to \textit{Pax}^{+/+};\textit{Ins2}^{+/+} littermates. The difference in mean glomerular area between the two genotypes was significant with a p-value of 0.0297.

\textbf{2.3.2.3 Diabetes leads to slow gradual increase in systolic blood pressure}

Hypertension is observed in early stages of DN progression in humans. To examine when hypertension begins to develop in diabetic mice with normal nephron numbers (\textit{Pax}^{+/+};\textit{Ins2}^{+/−}), we measured the systolic pressure of these mice between week 13 and 19. An overall trend of gradual increase in mean systolic pressure was observed in \textit{Pax}^{+/+};\textit{Ins2}^{+/−} mice between weeks 13 to 19, with a more profound increase observed between weeks 17 and 19. However, the mean blood pressures of \textit{Pax}^{+/+};\textit{Ins2}^{+/−} mice throughout weeks 13 to 19 were not significantly different from blood pressures of \textit{Pax}^{+/+};\textit{Ins2}^{+/+} littermates (Figure 2.10).
2.4 Diabetic mice show a marginal renal damage with slow progression towards DN

To examine the progression of DN between weeks 14 to 20 in litters from Pax2^{1Neu} and Ins2^{Akita}, urine samples were analyzed for albumin and creatinine concentrations. Subtle increase in ACR was observed overall in Pax^{+/+};Ins2^{+-} mice compared to Pax^{+/+};Ins2^{++} littermates between weeks 14 to 20 with significant increase in weeks 14 and 20 (p ≤ 0.05 and p ≤ 0.01, respectively) (Figure 2.11). Since hyperfiltration of the kidney precedes microalbuminuria in diabetic patients, we tested whether this is also the case for our diabetic mouse model. Change in GFR due renal impairment was estimated by measuring the amount of cystatin C in the serum of diabetic mice. By week 20, diabetes caused increase in GFR, a feature of early stage in DN, indicated by decrease in serum cystatin C in Pax^{+/+};Ins2^{+-} mice (n = 13) compared to Pax^{+/+};Ins2^{++} littermates (n = 20) (Figure 2.12).
Figure 2.11 Albuminuria is present in diabetic mice between weeks 14 and 20. An overall modest increase in UAE is present in \( \text{Pax}^{+/+};\text{Ins2}^{+/+} \) mice (n = 13) compared to \( \text{Pax}^{+/+};\text{Ins2}^{+/+} \) littermates (n = 18). The level of ACR increased approximately two-fold overall between weeks 14 and 20 (\( p = 0.0401 \)).

Figure 2.12 Increase in GFR is observed in diabetic mice by week 20. Hyperfiltration of the kidney is observed in \( \text{Pax}^{+/+};\text{Ins2}^{+/+} \) mice (n = 13) as indicated by a significant decrease in serum cystatin C compared to \( \text{Pax}^{+/+};\text{Ins2}^{+/+} \) littermates (n = 20).
2.3.3 Reduced nephron number accelerates the progression of DN in Pax<sup>+/−</sup>;Ins2<sup>+/−</sup>

2.3.3.1 Total nephron number and glomerular size in all genotypes of Pax2<sup>1Neu</sup> and Ins2<sup>Akita</sup> crosses

In our mouse model of generated by crossing Pax2<sup>1Neu</sup> and Ins2<sup>Akita</sup>, Pax2 mutation was the only factor that we are aware of that could lead to congenital nephron deficit. In order to confirm that this is the case, nephrons were counted in all four possible genotypes: 
Pax<sup>+/−</sup>;Ins2<sup>+/−</sup>, Pax<sup>+/−</sup>;Ins2<sup>+/−</sup>, Pax<sup>+/−</sup>;Ins2<sup>+/−</sup> and Pax<sup>+/−</sup>;Ins2<sup>+/−</sup>. The results show that the two genotypes carrying Pax2 mutations had reduced number of nephrons (Pax<sup>+/−</sup>;Ins2<sup>+/−</sup>: 22993 ± 3657, n = 3 and Pax<sup>+/−</sup>;Ins2<sup>+/−</sup>: 27933 ± 754.8, n = 3) in comparison to the two genotypes without Pax2 mutation (Pax<sup>+/−</sup>;Ins2<sup>+/−</sup>: 38544 ± 5765, n = 3 and Pax<sup>+/−</sup>;Ins2<sup>+/−</sup>: 41613 ± 2903, n = 3) (Table 2.1). This validates that Pax2 is the only factor associated with reduced nephron numbers in our mouse model.

Since both reduction in nephron number and diabetes compromise renal function, they are both associated with compensatory hypertrophy of the kidney. We assessed the glomerular size for all genotypes in our mouse model to examine how early the glomerular hypertrophy begins in mice with Pax2 or Ins2 mutation and whether the combination of the two mutations make the severity of glomerular hypertrophy worse. Our results demonstrate that the presence of Pax2 or Ins2 results in enlarged glomeruli (mean glomerular area in Pax<sup>+/−</sup>;Ins2<sup>+/−</sup>: 5967 ± 317.8 μm<sup>2</sup>, n = 3 and Pax<sup>+/−</sup>;Ins2<sup>+/−</sup>: 6586 ± 562.3 μm<sup>2</sup>, n = 3) compared to when neither of the mutations were present (Pax<sup>+/−</sup>;Ins2<sup>+/−</sup>: 4946 ± 279.8 μm<sup>2</sup>, n = 3). Mice with both Pax2 and Ins2 mutations (Pax<sup>+/−</sup>;Ins2<sup>+/−</sup>, n = 3) had the largest glomeruli with mean glomerular area of 6778 ± 341.2 μm<sup>2</sup>, which was significantly larger than glomerular area in Pax<sup>+/−</sup>;Ins2<sup>+/−</sup> (p = 0.0071) (Table 2.1).
Table 2.1 Mean nephron number and mean glomerular area in all four genotypes of $Pax^{21Neu}$ and $Ins^{2Kiita}$ crosses.

<table>
<thead>
<tr>
<th></th>
<th>Mean Nephron Number</th>
<th>Mean Glomerular Area (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Pax^{+/+};Ins2^{+/+}$ (n = 3)</td>
<td>38544 ± 5765</td>
<td>4946 ± 279.8</td>
</tr>
<tr>
<td>$Pax^{-/-};Ins2^{+/+}$ (n = 3)</td>
<td>22993 ± 3657</td>
<td>5967 ± 317.8</td>
</tr>
<tr>
<td>$Pax^{+/+};Ins2^{-/-}$ (n = 3)</td>
<td>41613 ± 2903</td>
<td>6586 ± 562.3</td>
</tr>
<tr>
<td>$Pax^{-/-};Ins2^{-/-}$ (n = 3)</td>
<td>27933 ± 754.8</td>
<td>6778 ± 341.2</td>
</tr>
</tbody>
</table>

2.3.3.2 Congenital nephron deficit accelerates the development of hypertension in diabetic mice

Brenner et al. postulated that individuals born with reduced nephron numbers are at higher risk of developing essential hypertension (Brenner et al., 1988). The association between diabetes and hypertension is also well-documented. We were curious to know whether congenital nephron deficit and diabetes together will have a combinatorial effect that will accelerate the development of hypertension. In order to test this, we measured systolic blood pressure in our mouse model at week 13 throughout week 19. We did not observe any dramatic increase in the blood pressure of $Pax^{+/+};Ins2^{+/+}$ (n = 17) and $Pax^{+/+};Ins2^{-/-}$ (n = 21) compared to $Pax^{+/+};Ins2^{+/+}$ (n = 23) (Figure 2.5 and Figure 2.10). There was a slow increasing trend that was observed in diabetic ($Pax^{+/+};Ins2^{+/+}$) mice, so we decided to compare it to $Pax^{+/+};Ins2^{+/+}$ to evaluate whether the presence of both $Pax2$ and $Ins2$ mutations will enhance the increase in blood pressure. The mean systolic blood pressure of $Pax^{+/+};Ins2^{+/+}$ mice (n = 16) remained consistently higher than blood pressure of $Pax^{+/+};Ins2^{+/+}$ mice at all time points between weeks 13 and 19 with an increasing trend ($p = 0.0122$) (Figure 2.13). The blood pressure of $Pax^{+/+}$
Ins2+/− mice was significantly higher than that of Pax+/−;Ins2+/− (p = 0.0281) and Pax+/−;Ins2+/+ (p = 0.0046) (data not shown). These results suggest that diabetic mice develop essential hypertension at a faster rate when they are born with reduced nephron number compared to those born with more nephrons.

Figure 2.13 Systolic blood pressure in Pax+/−;Ins2+/− vs Pax+/+;Ins2+/−. Diabetic mice with reduced nephron number (Pax+/−;Ins2+/−, n = 16) have higher blood pressure compared to diabetic mice with higher nephron number (Pax+/+;Ins2+/−, n = 21), p = 0.0122.

2.3.3.3 Suboptimal nephron number contributes to faster progression of DN in diabetic mice

In addition to the contribution of nephron numbers to the development of hypertension, Brenner et al. also suggested that reduction in congenital nephron number may also account for the susceptibility of diabetics to develop renal diseases (Brenner et al., 1988). In line with this idea, we aimed to test whether reduced nephron number will accelerate the progression of DN using our mouse model. UAE is one of the earliest biomarkers of DN and we calculated ACR using urine samples from our mouse model at weeks 14, 16, 18, 20. We observed that diabetic mice (Pax+/+;Ins2+/−) exhibited marginal increase in albuminuria between weeks 14 and 20. We
then examined to see if diabetic mice with reduced nephron numbers (Pax\(^{+/−}\);Ins2\(^{+/−}\)) will experience more severe albuminuria. Pax\(^{+/−}\);Ins2\(^{+/−}\) mice (n= 16) had subtle increase in urinary albumin excretion between weeks 14 and 18 but by week 20, these mice exhibited a striking increase in ACR (315.5 mg/g) which was significantly higher than ACR for the other genotypes (Pax\(^{+/+}\);Ins2\(^{+/+}\): \(p \leq 0.0001\), Pax\(^{+/−}\);Ins2\(^{+/+}\): \(p \leq 0.0001\), Pax\(^{+/+}\);Ins2\(^{+/−}\): \(p \leq 0.05\)) (Figure 2.14). These results demonstrate that reduced nephron number results in worse outcome in DN.

To evaluate the effect of nephron number on GFR in diabetic mice, cystatin C in serum samples collected from our mouse model at week 20 was quantified. Mean serum cystatin C concentration was similar in Pax\(^{+/+}\);Ins2\(^{+/+}\) and Pax\(^{+/−}\);Ins2\(^{+/+}\) littermates while serum cystatin C level was reduced in Pax\(^{+/+}\);Ins2\(^{+/−}\) mice (\(p = 0.0007\)). Mice carrying Pax2 and Ins2 mutations (Pax\(^{+/−}\);Ins2\(^{+/−}\)) exhibited serum cystatin C concentration (298.3 ± 28.74 ng/mL, n = 15) that was comparable to that of Pax\(^{+/−}\);Ins2\(^{+/+}\) (322.1 ± 16.24 ng/mL, n = 20) and Pax\(^{+/+}\);Ins2\(^{+/−}\) (326.0 ± 17.35 ng/mL, n= 15). Serum cystatin C concentration between Pax\(^{+/−}\);Ins2\(^{+/−}\) and Pax\(^{+/+}\);Ins2\(^{+/+}\) mice also showed no significant difference (Figure 2.15).
Figure 2.14 Albuminuria in all four genotypes of $Pax2^{1Neu}$ and $Ins2^{4kiia}$ crosses. Diabetic $Pax^{+/+};Ins2^{+/+}$ mice ($n = 15$) showed a marginal increase in albuminuria overall between weeks 14 to 20 compared to $Pax^{+/+};Ins2^{+/+}$ ($n = 18$) and $Pax^{+/+};Ins2^{+/+}$ mice ($n = 17$). $Pax^{+/+};Ins2^{+/+}$ mice ($n = 16$) exhibited subtle increase in microalbuminuria between weeks 14 and 18, but a dramatic increase in UAE was observed by week 20 relative to the other genotype littermates. (* $= p \leq 0.05$; ** $= p \leq 0.01$; **** $= p \leq 0.0001$, two-way ANOVA)

Figure 2.15 Serum cystatin C measured in all four genotypes of $Pax2^{1Neu}$ and $Ins2^{4kiia}$ crosses. Serum cystatin C in $Pax^{+/+};Ins2^{+/+}$ ($322.1 \pm 16.24$ ng/mL, $n = 20$) was almost identical to that of ($326.0 \pm 17.35$ ng/mL, $n = 15$), which were significantly higher than that observed in $Pax^{+/+};Ins2^{+/+}$ ($232.1 \pm 15.69$ N=13; $p = 0.0007$ and $p = 0.0005$ respectively). Serum cystatin C
in \( \text{Pax}^{+/\sim};\text{Ins2}^{+/\sim} \) mice (298.3 ± 28.74 ng/mL, \( n = 15 \)) were not significantly different from that of the other genotype littermates.

2.3.4 Rescue of nephron deficit by administration of Pro-VAD-FMK ameliorates renal impairment observed in \( \text{Pax}^{+/-};\text{Ins2}^{+/-} \) mice

Our lab has previously demonstrated that defective branching nephrogenesis due to \( \text{Pax2} \) mutations can be rescued with the treatment with a caspase inhibitor during renal development, which suppresses UB apoptosis observed in \( \text{Pax2}^{1\text{Neu}} \) mutant mice (Clark et al., 2004; Torban et al., 2000). In order to investigate whether the accelerated progression of DN observed in \( \text{Pax}^{+/-};\text{Ins2}^{+/-} \) mice is indeed a combinatorial effect of reduced nephron number caused by \( \text{Pax2} \) mutation and diabetes caused by \( \text{Ins2} \) mutation, we treated impregnated females carrying \( \text{Pax2}^{1\text{Neu}} \) and \( \text{Ins2}^{\text{Akita}} \) crosses with a caspase inhibitor, Pro-VAD-FMK from E13.5 to E20.5, when nephrogenesis takes place. We then assessed whether the characteristic features of renal damage observed in untreated \( \text{Pax}^{+/-};\text{Ins2}^{+/-} \) mice are rescued upon treatment with caspase inhibitor.

2.3.4.1 Increase in blood pressure observed in \( \text{Pax}^{+/-};\text{Ins2}^{+/-} \) mice is attenuated when defects in nephrogenesis is rescued

The effect of rescuing branching defect caused by \( \text{Pax2} \) mutations on blood pressure was investigated. \( \text{Pax2} \) mutation alone did not lead to the development of hypertension in the early adulthood in our mouse model and the blood pressure of these mice was comparable to that of their littermates with no mutation (\( \text{Pax}^{+/+};\text{Ins2}^{+/+} \)) throughout weeks 13 to 19, as shown previously in Figure 2.5. Since this suggests that renal damage in \( \text{Pax}^{+/-};\text{Ins2}^{+/-} \) mice is not severe enough to lead to essential hypertension at this point in life, we expected that rescue of nephrogenesis will not greatly impact the blood pressure in these mice. As expected, we observed no significant difference between the blood pressure of \( \text{Pax}^{+/-};\text{Ins2}^{+/-} \) mice that did not
receive any treatments and of same genotype mice that received Pro-VAD-FMK injections during embryogenesis (n = 6) (Figure 2.16). We also suspected that renal damage in diabetic mice (Pax+/−;Ins2+/−) was insufficient to cause a dramatic increase in blood pressure as suggested in Figure 2.10. Since the purpose of treatment with caspase inhibitor was to rescue the effect of Pax2 mutation on nephrogenesis, it should not cause any change in blood pressure in Pax+/−;Ins2+/− mice compared to that observed before the treatment. As anticipated, the blood pressure of Pax+/−;Ins2+/− mice that received Pro-VAD-FMK treatment (n = 12) showed the same slow increasing trend that was not significantly different from blood pressure of the same genotype mice without any treatments (Figure 2.17). The combinatorial effect of Pax2 and Ins2 was demonstrated by the early onset of hypertension in Pax+/−;Ins2+/− mice in Figure 2.13. To confirm that the effect of a Pax2 mutation on hypertension in Ins2+/− mice is mediated by loss of its anti-apoptotic effect, we reasoned that administration of an anti-apoptotic drug during embryonic life should ameliorate hypertension. Interestingly, treatment with caspase inhibitor (Pro-VAD-FMK) was able to rescue the hypertension observed in Pax+/−;Ins2+/− mice that were untreated (p = 0.0424) (Figure 2.18).
Figure 2.16 Systolic blood pressure in $Pax^{+/-};Ins2^{+/-}$ mice is unaffected by Pro-VAD-FMK treatment. Mean systolic blood pressures in untreated ($n = 17$) and Pro-VAD-FMK treated ($n = 6$) $Pax^{+/-};Ins2^{+/-}$ mice were not significantly different.

Figure 2.17 Systolic blood pressure in untreated $Pax^{+/-};Ins2^{+/-}$ mice and mice treated with Pro-VAD-FMK. The gradual increase in blood pressure observed in untreated $Pax^{+/-};Ins2^{+/-}$ mice ($n = 21$) was observed in $Pax^{+/-};Ins2^{+/-}$ mice treated with Pro-VAD-FMK ($n = 12$). No significant difference was observed between the mean blood pressure of untreated and treated mice.
Figure 2.18 Pro-VAD-FMK treatment attenuates hypertension observed in untreated \(\text{Pax}^{+/ -};\text{Ins}^{2+/ -}\) mice. Untreated \(\text{Pax}^{+/ -};\text{Ins}^{2+/ -}\) mice (n = 16) showed a significant increase \((p = 0.0122)\) in systolic blood pressure compared to untreated \(\text{Pax}^{+/ +};\text{Ins}^{2+/ -}\) mice (n = 21) between weeks 13 to 19. The significant difference between the two genotypes was not observed when systolic blood pressure of untreated \(\text{Pax}^{+/ +};\text{Ins}^{2+/ -}\) mice was compared to that of \(\text{Pax}^{+/ -};\text{Ins}^{2+/ -}\) mice treated with caspase inhibitor (n = 10). The blood pressure decreased significantly \((p = 0.0424)\) in \(\text{Pax}^{+/ -};\text{Ins}^{2+/ -}\) mice treated with Pro-VAD-FMK compared to the untreated \(\text{Pax}^{+/ -};\text{Ins}^{2+/ -}\) mice.

2.3.4.2 Signs of early DN is rescued in \(\text{Pax}^{+/ -};\text{Ins}^{2+/ -}\) mice treated with caspase inhibitor

We had observed a marked increase in UAE in \(\text{Pax}^{+/ -};\text{Ins}^{2+/ -}\) mice compared to the other genotype littermates at week 20 in Figure 2.14. We tested to see if this phenotype can be rescued by Pro-VAD-FMK treatment. We chose to compare untreated mice to treated mice at week 20 since week 20 showed the most dramatic increase in urinary albumin concentration in \(\text{Pax}^{+/ -};\text{Ins}^{2+/ -}\) mice. \(\text{Pax}^{+/ -};\text{Ins}^{2+/ -}\) mice treated with Pro-VAD-FMK (n = 7) demonstrated a 43.8% reduction in ACR compared to the same genotype mice with no treatment (n = 13). Although the difference was not statistically significant, the significant difference between untreated \(\text{Pax}^{+/ -};\text{Ins}^{2+/ -}\) and \(\text{Pax}^{+/ +};\text{Ins}^{2+/ -}\) mice observed in Figure 2.14 also diminished with Pro-VAD-FMK treatment. This suggests that rescue of nephrogenesis caused by \(\text{Pax}^{2}\) was able to reduce the
combinatorial effect of *Pax2* and *Ins2* mutations on UAE. Pro-VAD-FMK treatment did not cause significant changes in the other genotypes (Figure 2.19).

We then examined whether treatment with caspase inhibitor changes serum cystatin C levels in *Pax*+/+;*Ins2*+/− mice. Pro-VAD-FMK treatment caused significant decrease in serum cystatin C level in all genotypes (Figure 2.21). Serum cystatin C level remained similar between Pro-VAD-FMK-treated *Pax*+/+;*Ins2*+/+ and *Pax*+/−;*Ins2*+/+ mice as observed in untreated mice in Figure 2.7. *Pax*+/+;*Ins2*+/+ mice treated with Pro-VAD-FMK had serum cystatin C level that was significantly reduced compared to the other three genotype littermates that were also treated. *Pax*+/−;*Ins2*+/− mice treated with Pro-VAD-FMK has serum cystatin C level that was significantly lower than Pro-VAD-FMK-treated *Pax*+/+;*Ins2*+/+ mice (*p* = 0.0241) and higher than *Pax*+/+;*Ins2*+/− mice (*p* = 0.0173).

![Figure 2.19 Albumin-to-creatinine ratio (ACR) comparing *Pax21Neu* and *Ins24kita* crosses that received no treatments or Pro-VAD-FMK treatments at week 20. ACR in untreated and Pro-VAD-FMK-treated mice was not different for all four genotypes. There was a substantial amount of reduction in UAE in *Pax*+/−;*Ins2*+/+ mice treated with Pro-VAD-FMK (177.2 ± 76.60) at week 20.](image-url)
mg/g, n = 7) compared to the untreated $Pax^{+/+};Ins2^{+/-}$ mice (315.5 ± 60.78 mg/g, n = 13). The significant difference observed between $Pax^{+/-};Ins2^{+/-}$ and $Pax^{+/-};Ins2^{+/-}$ mice was no longer present when the mice received Pro-VAD-FMK treatment.

Figure 2.20 Serum cystatin C comparing $Pax2^{1Neu}$ and $Ins2^{Akita}$ crosses that received no treatments or Pro-VAD-FMK treatments at week 20. Treatment with Pro-VAD-FMK caused significant reduction in serum cystatin C level in all genotypes compared to the untreated mice. (** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$)

2.4 Discussion

Diabetic nephropathy is the most prevalent and severe complications of diabetes, affecting millions of people globally. DN progresses in stages with the initial stage characterized by relatively low levels of microalbuminuria, which gradually increases until the kidney becomes severely damaged and experiences extensive proteinuria. Hence, the onset of albuminuria marks the early phases of DN and is often accompanied the development of hypertension. Hypertension is one of the major contributor to renal deterioration in DN as it substantially increases the risk of macrovascular and microvascular complications. Prolonged renal injury in late stages of DN compromises kidney function and results in reduced glomerular
filtration rate (GFR) and eventually leads to ESRD. Progression of DN largely varies between patients but a subset of diabetic patients has been shown to exhibit rapid loss of renal function. These “fast decliners” account for the majority of end-stage renal disease cases in type I diabetes (Krolewski et al., 2017), but factors that predisposes a subset of diabetic patients to a more accelerated progression of DN remains unknown. Therefore, identifying diabetic patients that are more susceptible to rapid renal deterioration may prevent or delay the progression of nephropathy.

There is a wide variation in nephron number in humans, ranging from 210,000 to 2,700,000 (Bertram et al., 2011). In 1988, Brenner et al. proposed that congenital nephron deficit may be the explanation to why some diabetic individuals are more likely to develop hypertension and renal diseases. The final nephron endowment is determined by the branching of UB during kidney development in fetal life. The renal-coloboma syndrome (RCS) is a rare autosomal dominant disorder in humans that is characterized by renal hypoplasia and optic nerve colobomas (Eccles & Schimmenti, 1999; Sanyanusin et al., 1995). Mutations of a developmental gene involved in nephrogenesis, PAX2 have been identified to be the cause of RCS. Further studies of PAX2 haploinsufficiency in human population have been limited due to impracticality of obtaining kidney samples. However, a mouse strain (Pax21Neur) that carries the same mutation has been discovered and heterozygous Pax2 mutation exhibited optic nerve and kidney anomalies similar to humans with RCS (Favor et al., 1996). Studies using this mouse strain have demonstrated that Pax2 has an anti-apoptotic role in the growing UB cell lineage and that loss of Pax2 anti-apoptotic activity results in reduced UB branching and hence nephron formation during kidney development (Clark et al., 2004; Dziarmaga et al., 2003; Porteous et al., 2000).
In this study, we demonstrated *in vivo* the association between nephron number and susceptibility to hypertension and renal diseases. We crossed a mouse strain that exhibits reduced congenital nephron number (\textit{Pax2}^{\text{1Neu}}) with a mouse model for type I diabetes (\textit{Ins2}^{\text{Akita}}) and assessed parameters associated with DN. To account for any variations that may result due to strain differences, only the littermates of the crossed strain were used as controls. The effect of heterozygous \textit{Pax2} mutation on nephron number was confirmed in our crossed strain. Congenital nephron number was reduced in \textit{Pax2}^{+/--};\textit{Ins2}^{+/+} and \textit{Pax2}^{+/--};\textit{Ins2}^{+/+} mice compared to their littermates without \textit{Pax2} mutation (\textit{Pax2}^{++/+};\textit{Ins2}^{+/+} and \textit{Pax2}^{++/+};\textit{Ins2}^{+/+}). Although the reduction in nephron number was not statistically significant in \textit{Pax2}^{++/+};\textit{Ins2}^{+/+} mice compared to \textit{Pax2}^{++/+};\textit{Ins2}^{++/+} littermates, 27.5% reduction in nephron number was observed. The non-significant result may be due to the small sample size (n = 3) but congenital nephron deficit was evident in \textit{Pax2}^{++/+};\textit{Ins2}^{++/+} mice nonetheless.

Since both nephron deficit and diabetes compromise renal function and are associated with compensatory hypertrophy, we postulated that mice carrying at least one of \textit{Pax2} or \textit{Ins2} mutations may have enlarged glomeruli. As expected, we observed increased glomerular size in mice with \textit{Pax2} or \textit{Ins2} mutations compared to wildtype (no mutation) littermates, and mice carrying both mutation showed the largest increase in glomerular size. We also observed early onset of DN in mice with both \textit{Pax2} and \textit{Ins2} mutations (\textit{Pax2}^{++/+};\textit{Ins2}^{+/+}) indicated by increased blood pressure and albuminuria compared to their littermates. There are many factors that contribute to normal, small blood pressure fluctuations from day to day and to account for this fluctuation, we measured blood pressure twice a week in our mice. Even with biweekly blood pressure measurements, the blood pressure fluctuated during the study period (week 13 to week 19) and therefore, we decided to focus on observing the trend of the blood pressure.
measurements. Whereas the blood pressure remained steady and in the normal range in $Pax2^{+/+};Ins2^{+/+}$ and $Pax2^{+/+};Ins2^{+/+}$ mice, a gradual increase was observed in diabetic mice ($Pax2^{+/+};Ins2^{+/+}$). We were expecting to observe a slight increase in blood pressure in mice with $Pax2$ mutation alone due to compromised nephron number, as Brenner et al. also hypothesized. However, we believe it will take longer for these mice with reduced nephron numbers to develop hypertension. In comparison, $Pax2^{+/+};Ins2^{+/+}$ mice was showing high systolic blood pressure compared to the other genotype littermates by week 13 and showed an increasing trend in blood pressure measurements. Even with the fluctuations in blood pressure throughout the weeks, $Pax2^{+/+};Ins2^{+/+}$ mice demonstrated significantly higher blood pressure compared to the other genotype littermates at all points between weeks 13 to 19. This result suggests that although $Pax2$ or $Ins2$ mutation alone is insufficient to cause significant increase in blood pressure during relatively early phase of nephropathy, the two mutations together accelerate the development of hypertension. We also observed this combinatorial effect of reduced nephron number and diabetes in albuminuria. $Pax2^{+/+};Ins2^{+/+}$ and $Pax2^{+/+};Ins2^{+/+}$ mice did not show signs of albuminuria throughout weeks 14 to 20 and $Pax2^{+/+};Ins2^{+/+}$ mice showed marginal effect on albuminuria with increased ACR observed in weeks 14 and 20, but not in weeks 16 and 18, which may be a result of biological variation. Similar to the blood pressure results, combination of $Pax2$ and $Ins2$ mutations was associated with a substantial increase in UAE with a progressive increase starting from week 14 throughout week 18 and by week 20, $Pax2^{+/+};Ins2^{+/+}$ mice demonstrated a dramatic increase in ACR compared to the other genotype littermates.

Association between congenital nephron deficit and susceptibility to hypertension and albuminuria was further confirmed by treating the mice with a caspase inhibitor, Pro-VAD-FMK. Treatment with a caspase inhibitor during nephrogenesis in fetal life has been shown to rescue
the anti-apoptotic activity in the growing UB and branching defects caused by Pax2 mutations (Clark et al., 2004; Torban et al., 2000). In our in vivo study, treatment with Pro-VAD-FMK rescued the increase in hypertension and albuminuria observed in Pax2+/−;Ins2+/− mice, which validates the effect of reduced nephron number on early manifestation of DN.

To estimate the effect of reduced nephron number and diabetes in renal function, we estimated GFR by measuring serum cystatin C in our Pax21Neu and Ins2Akitat crossed mouse strain. We observed a decrease in serum cystatin C in diabetic mice (Pax2+/−;Ins2+/−) compared to Pax2+/+;Ins2+/+ and Pax2+/+;Ins2+/− littermates, which possibly reflects the surge in GFR observed in the early phase of DN. Serum cystatin C level in Pax2+/−;Ins2+/− mice was between that of Pax2+/+;Ins2+/+ and Pax2+/+;Ins2+/− mice and Pax2+/+;Ins2+/− mice. Whether this result reflects the GFR dropping back down after the surge in GFR, resembling the normal filtration rate observed in incipient DN, or the GFR before it begins to surge cannot be determined. Further studies with a longer study period investigating the association between reduced nephron number and GFR will be able to confirm the effect of nephron deficit and diabetes on GFR. Interestingly, we observed a significant decrease in serum cystatin C level in all four genotypes of the crossed strain. Previous study has shown that treatment with a caspase inhibitor not only increased branching activity in the Pax21Neu mutant kidneys, but also increased terminal branch number in the wild-type kidneys albeit to a lesser degree (Clark et al., 2004). This explains the decrease in serum cystatin C in all genotypes treated with Pro-VAD-FMK.

In conclusion, our in vivo study using Pax21Neu and Ins2Akitat cross mouse strain demonstrate that Pax2 haploinsufficiency in Pax21Neu strain contributes to accelerated development of DN, indicated by increase in systolic blood pressure and albuminuria by week 20 of age. Due to the limitations in studying the direct effect of Pax2 mutation and congenital
nephron deficit on progression of DN in humans, our mouse crosses serve as an ideal model of DN progression in humans born with low nephron numbers. These observations strongly support the hypothesis that congenital nephron deficit results in early manifestations of DN.
Our mouse data suggest that the presence of heterozygous null $Pax2$ mutation ($Pax2^{1Neu}$) exacerbates the progression of albuminuria and hypertension, signs of early diabetic nephropathy (DN), in diabetic mice ($Ins2^{Akita}$). During this study period, a relatively early time frame for DN progression, loss of GFR was not observed, which usually occurs at a later stage in DN. Brenner et al. hypothesized that in the human population, normal individuals born with low nephron numbers will show accelerated renal damage in setting of diabetes (Brenner et al., 1988). Our in vivo study using $Pax2^{1Neu}$ and $Ins2^{Akita}$ crosses demonstrated in Chapter II supports this hypothesis. In the following chapter of my thesis, we studied the association between reduced nephron number and susceptibility to DN in human diabetic cohort using common $PAX2$ variants which were shown to compromise kidney volume, a surrogate for nephron number. We postulated that in our diabetic cohort, we may see early signs of albuminuria and hypertension in patients with $PAX2$ SNPs, as we did in our in vivo study. We did not expect to see any change in GFR in our cohort, which is normally observed in late stages of DN.
CHAPTER III: Common polymorphisms in the PAX2 gene associated with renal hypoplasia lead to early onset of diabetic nephropathy in diabetic cohort
3.1 Overview

*PAX2* gene, which encodes an important transcription factor that regulates nephrogenesis, is located on chromosome 10 at band 10q24 in humans and consists of 12 exons spanning over 70 Kb of genomic DNA (Narahara et al., 1997; Sanyanusin et al., 1996). A severe congenital nephron deficit is observed in patients with *PAX2* haploinsufficiency. In renal-coloboma syndrome (RCS), a rare autosomal dominant condition caused by heterozygous mutation in *PAX2* gene, a remarkable decrease in the absolute nephron number is reported (Sanyanusin et al., 1995). Recently, a common variant of the *PAX2* gene was identified to be associated with a subtler reduction in nephron number (Quinlan et al., 2007). These single-nucleotide polymorphisms (SNPs), clustered in a region that spanned intron 3 to intron 5 of the *PAX2* gene, were linked to reduced kidney volume in healthy newborns and allele-specific *PAX2* mRNA level in human renal cell carcinoma cell lines. In particular, a common *PAX2* haplotype (AAA), constructed using three of the identified SNPs that were in tight linkage disequilibrium (LD), was shown to occur in 18.5% of the newborn cohort and was associated 10% reduction in kidney volume. In the human population, congenital nephron number varies greatly across individuals and is observed to be normally distributed (Nyengaard & Bendtsen, 1992). Since these polymorphisms are relatively common and contribute to subtle renal hypoplasia in normal newborns, they may represent one of the multiple variables that accounts for low nephron numbers in the normal population.

There is a growing interest in identifying risk factors that contribute to the pathogenesis of diabetic nephropathy at an early stage. Studies have suggested that both environmental factors and genetic susceptibility play important roles in the development and progression of diabetic nephropathy (Diabetes et al., 1993; Quinn, Angelico, Warram, & Krolewski, 1996), making
identification of susceptibility genes for diabetes and DN complicated. While DN takes decades after the onset of diabetes to progress onto ESRD, some diabetic patients have been shown to experience accelerated renal decline towards ESRD (Krolewski et al., 2017). Several genome-wide studies have attempted to identify genes associated with DN, but they were not successful in identifying loci with dramatic clinical effects (Tanaka & Babazono, 2005; Teumer et al., 2016). Discovering markers that can predict the rate of renal decline is crucial for the detection and treatment of diabetic patients that are at a higher risk of early onset of ESRD.

As Brenner et al. hypothesized, congenital nephron deficit may account for increased susceptibility to acquired renal disease and the development of hypertension (Brenner et al., 1988). We propose that PAX2 variants that are associated with mild form of renal hypoplasia may predispose individuals with these SNPs to fast progression of DN. To investigate the association between common PAX2 SNPs and susceptibility to renal damage, we performed an imputation-based association analysis for four of the PAX2 variants discovered to contribute renal hypoplasia by Quinlan et al. (Quinlan et al., 2007). Individuals who carry at least one of these PAX2 variants in our young diabetic cohort were identified using SNP imputation. These individuals were then assessed for clinical signs of DN, such as albuminuria, hypertension and elevated serum creatinine level and compared with individuals in the cohort that do not carry these PAX2 SNPs.

3.2 Materials and Methods
3.2.1 Study Subjects
Population cohort of children diagnosed with Type I diabetes at Montreal’s Children Hospital (MCH) was obtained from Dr. Constantin Polychronakos. All patients of European decent were recruited from Montreal, Canada with informed consent form signed by the patients
or their parents. Within this cohort, 212 patients had genome-wide association study (GWAS) data and were used in this study. These subjects were between the age of 13 and 37 years. The study (MCH-001-20) was approved by the MCH Institutional Review Board.

3.2.2 Clinical data

Most recent clinical information of genotypes of patients in the diabetic cohort (urinary albumin-to-creatinine ratio, blood pressure and serum creatinine level) was gathered using the electronic medical record system, OACIS provided by McGill University Health Centre (MUHC).

3.2.3 Imputation

Since the PAX2 gene is located on chromosome 10 in humans, SNPs that span chromosome 10 were extracted from our GWAS data using gPLINK, a java-based software package. PLINK (v.1.9), a whole genome association analysis toolset, was used to convert the genotypes from 012 format to the original phased genotype format and to convert .map and .ped files into .vcf files for further processing. Since our data was based on the older human reference genome assembly, NCBI35/hg17, it was converted to a newer version (GRCh37/hg19) using CrossMap 0.2.8 program. SNPs in chromosome 10 were imputed using a computer server, Michigan Imputation Server. The imputed file was then converted to plink file using VCFtools program from which our SNPs of interest (rs11599825, rs11190688, rs11190702 and rs1800898) were extracted using PLINK (v.1.9).

3.3 Results

3.3.1 Descriptive study of early DN in type I diabetic cohort

Quinlan et al. identified variants of the PAX2 gene that are associated with subtle reduction in kidney size (Quinlan et al., 2007). We postulated that presence of these SNPs will
result in early manifestation of DN. To test our hypothesis, we assembled a small cohort of young patients with type I diabetes who had undergone GWAS analysis and reviewed their clinical data to assess features associated with DN. Out of the 212 patients with GWAS data in our cohort, 156 patients had information on ACR, 92 patients had blood pressure data and 105 patients had serum creatinine data. The most recent data for each of the parameters was collected and analyzed.

Patient information was analyzed for the duration of diabetes when the most recent clinical data was recorded (Table 3.1). Duration of diabetes in these patients ranged between 0 to 29 years. Since our diabetic cohort consists of young patients, the majority of the patients have been diagnosed with diabetes for 15 years or less. Distribution of the patients based on each of the parameters was also analyzed for the purpose of examining the distribution amongst diabetic patients and how many of the patients have signs of DN. Histogram of urinary ACR in our cohort appeared to form a normal distribution, which demonstrated that 92.9% of the patients had ACR less than or equal to 4.0 mg/mmol (Figure 3.1). 11 outliers were observed to fall outside of the distribution with ACR value greater than 5.0 mg/mmol. We postulated that these individuals may represent the population with PAX2 variants who are born with low nephron numbers. Frequency distribution of systolic blood pressure data in diabetic patients was skewed to the right (Figure 3.2). Both the mean and the median values were 115 mmHg and the 95% confidence interval (CI) was between 113 and 117 mmHg. The minimum value was 90 mmgHg and the maximum value was 156 mmHg. Frequency distribution of serum creatinine data of diabetic cohort displays a bimodal distribution with the two peaks occurring between the ranges 57.5-62.5 µmol/L and 77.5-82.5 µmol/L (Figure 3.3). Since serum creatinine level is dependent on gender, we postulated that the bimodal distribution may be a result of the variability in males
and females. To validate this, we plotted separate histograms of serum creatinine for male and female diabetic patients. Creatinine distributions in males appeared to be irregularly distributed whereas in females it appeared to be normally distributed with a slight skew to the right. As predicted statistical values were different between the two groups. In the male cohort (n = 52), the mean was 72.1 µmol/L, the median was 74.0 µmol/L and the 95% CI was 67.9-76.4 µmol/L (Figure 3.4). In contrast, the female cohort (n = 53) had a mean value of 58.1 µmol/L, mean value of 57.0 µmol/L and 95% CI of 54.4-61.7 µmol/L (Figure 3.5). These values were in accordance with the normal serum creatinine ranges reported by MUHC Laboratory Reference ranges (MUHC, Montreal, Canada); males: 55-110 µmol/L, females: 40-85 µmol/L, suggesting majority of the diabetic patients in the cohort have not developed DN yet.

Table 3.1 Duration of diabetes in type I diabetic cohort with albuminuria, blood pressure and serum creatinine data.

<table>
<thead>
<tr>
<th>Duration of Diabetes</th>
<th>0-5 years</th>
<th>6-10 years</th>
<th>11-15 years</th>
<th>16-20 years</th>
<th>&gt; 20 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albuminuria (n = 156)</td>
<td>13 patients</td>
<td>61 patients</td>
<td>56 patients</td>
<td>20 patients</td>
<td>6 patients</td>
</tr>
<tr>
<td>Blood Pressure (n = 92)</td>
<td>1 patient</td>
<td>14 patients</td>
<td>43 patients</td>
<td>21 patients</td>
<td>13 patients</td>
</tr>
<tr>
<td>Serum Creatinine (n = 105)</td>
<td>12 patients</td>
<td>27 patients</td>
<td>37 patients</td>
<td>20 patients</td>
<td>9 patients</td>
</tr>
</tbody>
</table>
Figure 3.1 Frequency distribution of type I diabetic cohort based on urinary albumin-to-creatinine ratio (ACR) data. 92.9% of the urinary ACR values fell under 5.0 mg/mmol with a median value of 0.65 mg/mmol and mean value 1.93 mg/mmol. 11 values out of 156 fell outside of the half-normal distribution (> 5.0 mg/mmol). The maximum value was 39.7 mg/mmol.
Figure 3.2 Frequency distribution of type I diabetic cohort based on systolic blood pressure data. The minimum value was 90 mmHg and the maximum value was 156 mmHg. 95% confidence interval was 113-117 mmHg. The mean and the median values were both 115 mmHg. Total number of values analyzed in the distribution was 92.

Figure 3.3 Frequency distribution of serum creatinine in type I diabetic cohort. Serum creatinine level in the diabetic patients exhibits bimodal distribution. The minimum value was 34 µmol/L and the maximum was 131 µmol/L.
Figure 3.4 Frequency distribution of serum creatinine in male type I diabetic cohort. Serum creatinine level in male diabetic cohort (n = 52) exhibits irregular distribution. Mean = 72.1 µmol/L, median = 74.0 µmol/L, 95% CI = 67.9-76.4 µmol/L.

Figure 3.5 Frequency distribution of serum creatinine in female type I diabetic cohort. Serum creatinine level in female diabetic cohort (n = 53) displays a normal distribution slightly skewed to the right. Mean = 58.1 µmol/L, median = µmol/L, 95% CI = 54.4-61.7 µmol/L.
3.3.2 SNP imputation and association studies

Out of the five $PAX2$ variants associated with subtle renal hypoplasia discovered by Quinlan et al., we chose to study four SNPs that are relatively common that are tightly linked to each other and resulted in reduced $PAX2$ transcript level. Since these $PAX2$ variants were not present in our GWAS database, our SNPs of interest (rs11599825, rs11190688, rs11190702 and rs1800898) had to be imputed using human reference genome to infer the alleles of our ‘hidden’ SNPs. Individuals in our diabetic cohort with our SNPs of interest were identified and their alleles were analyzed to further sort out patients who are heterozygous or homozygous for the less common allele associated with reduced kidney volume and reduced $PAX2$ transcript level: rs11599825 (A), rs11190688 (A), rs11190702 (A) and rs1800898 (C). We examined whether individuals carrying these $PAX2$ SNPs associated with renal hypoplasia were more susceptible to early manifestation of DN based on their clinical data.

3.3.2.1 $PAX2$ variants are associated with susceptibility to albuminuria in diabetic patients

Generally, urinary albumin-to-creatinine ratio (ACR) greater than 2.5-3.5 mg/mmol is considered microalbuminuria (guidelines from The Renal Association and Kidney Health Australia). The distribution of our diabetic cohort based on their urinary ACR data demonstrated that the population seemed to form a half-normal distribution with potential outliers lying above ACR level of 5 mg/mmol (refer to Figure 3.1). We were particularly interested in these patients at the higher end because they represent a small group of diabetic patients with early signs of DN compared to the rest of diabetic population. We hypothesized that this small group of diabetic patients exhibiting higher ACR compared to most diabetics may be associated with our SNPs that are linked to low nephron numbers. To test this hypothesis, we first set ACR value that distinguishes between albuminuria and no albuminuria to be 5 mg/mmol based on our frequency
distribution. Since overt diabetic nephropathy begins at around 15 years since the diagnosis of diabetes, when most diabetic patients begin to develop albuminuria, we focused on patients that have been diagnosed with diabetes for 15 years or less to study the effect of \textit{PAX2} variants on early manifestation of DN. Out of 156 diabetic patients with ACR data, 130 patients have been diagnosed with diabetes for 15 years or less at the most recent follow-up and among them, 28 had at least one of our \textit{PAX2} SNPs. To determine whether this SNP is associated with early onset of albuminuria, we made a contingency table and analyzed the association between \textit{PAX2} SNPs and albuminuria (Table 3.2). The association between \textit{PAX2} SNPs and albuminuria was significant ($p = 0.0376$) with the relative risk (RR) of 4.22, suggesting diabetic patients with \textit{PAX2} SNPs associated with reduced nephron number are more susceptible to albuminuria compared to diabetic patients without these SNPs. Interestingly, 5 out of 6 diabetic patients in the cohort ($n = 156$), who experienced severe albuminuria of greater than 10 mg/mmol were carriers of \textit{PAX2} SNPs and 4 of them have been diagnosed with diabetes for 15 years or less and exhibited ACR greater than 20 mg/mmol. This further supporting the association between congenital nephron deficit and early manifestation of DN.

Table 3.2 Contingency table demonstrating the association between \textit{PAX2} SNPs and albuminuria in patients who have been diagnosed with diabetes for 15 years or less. (Fisher’s exact test: $p = 0.0376$)

<table>
<thead>
<tr>
<th></th>
<th>Albuminuria</th>
<th>No Albuminuria</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ \textit{PAX2} SNPs</td>
<td>5</td>
<td>23</td>
<td>28</td>
</tr>
<tr>
<td>- \textit{PAX2} SNPs</td>
<td>5</td>
<td>97</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>120</td>
<td>130</td>
</tr>
</tbody>
</table>
3.3.2.2 Association between PAX2 SNPs and hypertension

Hypertension is a common condition associated with diabetes, affecting approximately 20-60% of diabetic patients. It is also considered an indicator of onset of DN in type I diabetes that subsequently leads to increased susceptibility to macrovascular and microvascular complications observed in DN (Arauz-Pacheco, Parrott, Raskin, & American Diabetes, 2003). Brenner et al. hypothesized that reduced nephron number may contribute to increased risk of developing hypertension (Brenner et al., 1988) and our in vivo study using mice demonstrated that blood pressure was significantly elevated in diabetic mice with congenital nephron deficit (Chapter II of this thesis). Taken together, we postulated that common PAX2 variants associated with reduced nephron number may contribute to higher risk of developing hypertension in diabetic patients. According to American Heart Association guideline for high blood pressure, systolic blood pressure greater than 130 mmHg is considered hypertensive. Detection of hypertension is different in young patients who are less than 18 years old. Blood pressure is reported in percentiles using a calculator that is based on gender, age and height. Based on the results from the calculator, blood pressure in less than 90th percentile is considered normal, between 90th and 95th percentile is considered pre-hypertensive and greater than or equal to 95th percentile is considered hypertensive. We set systolic blood pressure >130 mmHg as hypertensive and greater than 95th percentile as hypertensive for children in our diabetic cohort. We then analyzed the association between imputed PAX2 SNPs and hypertension in patients who have been diagnosed with diabetes for 15 years or less (n = 58) using a contingency table (Table 3.3). The association was not significant, but it is interesting to note the relative risk of having PAX2 SNPs and being hypertensive, which was 2.4.
Table 3.3 Contingency table demonstrating the association between \( PAX2 \) SNPs and hypertension. The association between \( PAX2 \) SNPs and hypertension was not significant, but the corresponding relative risk was 2.4.

<table>
<thead>
<tr>
<th></th>
<th>Hypertensive</th>
<th>Normotensive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ ( PAX2 ) SNPs</td>
<td>11</td>
<td>23</td>
<td>14</td>
</tr>
<tr>
<td>- ( PAX2 ) SNPs</td>
<td>4</td>
<td>40</td>
<td>44</td>
</tr>
</tbody>
</table>

3.3.2.3 Association between \( PAX2 \) SNPs and serum creatinine

The onset of DN, usually defined by the onset of microalbuminuria, is followed by a slow gradual decline in glomerular filtration rate (GFR). GFR is the best parameter of kidney function in diabetic patients and serum creatinine is a commonly used marker for estimating kidney function and GFR. Since serum creatinine is dependent on age and gender, separate reference ranges are used to define high creatinine level in patients. For males older than 18 years of age, serum creatinine greater than 110 µmol/L is considered high, for females older than 18 years of age, serum creatinine greater than 85 µmol/L is considered high (MUHC-RI Laboratory reference range). For patients under the age of 18 years, reference range differs by age and gender. Using these reference ranges, we identified patients who show high serum creatinine level and examined the association between \( PAX2 \) SNPs and high creatinine in patients who have been diagnosed with diabetes for 15 years or less (n = 76) using a contingency table (Table 3.4). We did not find significant association between our \( PAX2 \) SNPs and high creatinine. Relative risk having \( PAX2 \) SNPs and high creatinine was 1.6.
Table 3.4 Contingency table demonstrating the association between \(PAX2\) SNPs and high creatinine. No significant association between \(PAX2\) SNPs and high serum creatinine was observed. Reported relative risk was 1.6.

<table>
<thead>
<tr>
<th></th>
<th>High creatinine</th>
<th>Normal creatinine</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ (PAX2) SNPs</td>
<td>5</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>- (PAX2) SNPs</td>
<td>8</td>
<td>47</td>
<td>55</td>
</tr>
</tbody>
</table>

### 3.4 Discussion

In humans, congenital nephron number is normally distributed and a wide variation in nephron number is observed ranging from 210,000 to 2,700,000 (Bertram et al., 2011). There are likely multiple genetic factors that determine the final congenital nephron number. Mutations in \(PAX2\) have been identified in patients diagnosed with renal-coloboma syndrome (RCS) or oligomeganephronia, which are disorders associated with severe renal hypoplasia (Sanyanusin et al., 1995). The importance of \(Pax2\) in nephrogenesis is also evident in a mouse strain harboring a spontaneous \(Pax2\) mutation (\(Pax2^{1Neu}\)), in which heterozygous mutation in \(Pax2\) gene results in renal anomalies and reduced nephron number (Favor et al., 1996). A direct role of \(Pax2\) in suppressing UB apoptosis and hence, in the survival of UB cells during nephrogenesis has been demonstrated (Clark et al., 2004; Dziarmaga et al., 2003; Torban et al., 2000). Although severe renal hypoplasia, a surrogate for reduced nephron number, observed in patients diagnosed with RCS and oligomeganephronia may account for some individuals with suboptimal nephron numbers, normal individuals born with lower nephron numbers needed to be considered. Quinlan
et al. identified common polymorphisms in the PAX2 gene associated with subtle renal hypoplasia in normal individuals (Quinlan et al., 2007).

Brenner et al. suggested that congenital nephron deficit may contribute to susceptibility to development of hypertension and renal disease in diabetic patients. Diabetic nephropathy (DN), the most prevalent and severe complication of diabetes, generally takes many years to develop and progresses in stages, with each stage marked by different clinical outcomes. Studies have reported some diabetic patients experience a rapid decline in renal function and accelerated progression in DN (Krolewski et al., 2017). We postulated that individuals born with PAX2 SNPs associated with renal hypoplasia are these fast decliners that exhibit faster progression in DN.

We tested our hypothesis by examining the association between PAX2 SNPs and clinical outcomes associated with DN in a cohort of type I diabetic patients. We observed a significant association between PAX2 SNPs and early onset of albuminuria in patients who have been diagnosed with diabetes for 15 years or less ($p = 0.0376, RR = 4.2$). Our results demonstrated that individuals who are carriers of PAX2 SNPs are more likely to experience severe albuminuria at a relatively early stage in DN. However, PAX2 SNPs were not shown to be significantly associated with hypertension and high serum creatinine in patients who have been diagnosed with diabetes for 15 years or less. Interestingly, relative risk higher than 1 was observed for both cases. Some of the possible explanations for this outcome may be that time frame of less than 15 years since the diagnosis of diabetes may be too early for hypertension and high serum creatinine to arise, and that the sample size was simply too small. Some of the drawbacks in our study were that the number of GWAS data and the corresponding clinical information was very limited. Sample sizes used in all three association studies were too small to be powered to draw
conclusion about the impact of *PAX2* SNPs on our clinical data (albuminuria, blood pressure, serum creatinine). Therefore, a study with a larger number of diabetic patients with clinical data will further validate our results. In addition, treatments for each diabetic patient was not accounted for in our study. Taking treatments into consideration will further improve the outcomes of this study.
Conclusions and Future Directions

It has long been suggested that congenital nephron deficit may be associated with susceptibility to the development of hypertension and acquired renal disease, but there haven’t been any studies proving this hypothesis. Our \textit{in vivo} study using $Pax2^{lnNeu}$ and $Ins2^{Akita}$ crossed mouse strain provides a direct evidence for the association between nephron number and progression of DN. Both congenital nephron deficit and diabetes are factors that contribute to the development of essential hypertension and compromise renal function. Using our mouse model, we were able to demonstrate that when the effects of reduced nephron number and diabetes on renal function are combined, renal damage progresses faster. It was observed that mice with both $Pax2$ and $Ins2$ mutations developed hypertension and albuminuria at an early stage even before these signs of DN were apparent in mice with either $Pax2$ or $Ins2$ mutation alone. We were also able to further validate the contribution of suboptimal nephron number to early manifestation of DN by rescuing the branching defects caused by $Pax2$ mutation using a caspase inhibitor. In $Pax2^{+/-};Ins2^{+/-}$ mice treated with caspase inhibitor, the combinatorial effect of $Pax2$ and $Ins2$ mutations was diminished and the systolic blood pressure and urinary albumin excretion was reduced to levels similar to that observed in diabetic ($Pax2^{+/-};Ins2^{+/-}$) mice.

Since DN progresses at a faster rate in a subset of individuals, we postulated that human diabetic patients who are born with reduced nephron number may represent these “fast-decliners”. We tested to see whether common variants of $PAX2$ gene identified to be associated with subtle renal hypoplasia in normal individuals result in early manifestation of DN when these individuals are diagnosed with diabetes later in life. We demonstrated that diabetic patients with these $PAX2$ SNPs are more likely to develop albuminuria at an early stage of DN. However, presence of $PAX2$ SNPs did not significantly increase the likelihood of developing hypertension.
or increasing serum creatinine level in our diabetic patients. There are definitely areas for improvement in this study, such as increasing the number of study subjects, which will be able to validate our results on the association between $PAX2$ SNPs and biomarkers of DN.

Taken together, our $in\ vivo$ and association studies demonstrate that congenital nephron deficit accelerates the progression of early markers of DN, such as albuminuria. This study has implications for the pathogenesis of DN in diabetic patients with reduced nephron number. If our observations can be confirmed in larger diabetic cohorts, it may suggest a method by which the risk for accelerated nephropathy can be identified at the onset of disease, allowing for intensified treatments in selected individuals.
REFERENCES


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