Hypertrophy of Human Renal Glomeruli: Links with Disease Risk Factors and Podocyte Depletion

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

by

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May 2014
Notice 1
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SUMMARY

Chronic kidney disease (CKD) is a global pandemic associated with increasingly high health care costs and great burden of disease. Once CKD is established, it usually progresses to end stage kidney disease (ESKD), which requires dialysis or renal transplantation as life-saving interventions. In order to develop preventive strategies for CKD, a better understanding of the structural changes that occur in kidneys in pre-disease stages is needed. The studies described in this thesis constitute a rigorous analysis of human glomerular volume (and thereby glomerular hypertrophy) in subjects without renal disease, and its association with: (1) multiple CKD risk factors; and (2) cellular components of these hypertrophic glomeruli, with a focus on podocytes. The general hypothesis of this thesis, as reviewed in Chapter 1, is that “glomerular hypertrophy is multifactorial, compensatory and associated with relative podocyte depletion in subjects without renal disease”.

Glomerular hypertrophy is classically viewed as a result of a nephron deficit. However, Chapters 2 and 3 challenge this perception by introducing the concept of multifactorial glomerular hypertrophy. Chapter 2 shows that multiple risk factors for CKD (including older age, obesity and hypertension) are associated with higher degrees of glomerular hypertrophy than is observed in subjects with one or no CKD risk factors. Chapter 3 provides evidence of greater glomerular hypertrophy in African Americans in the context of older age, lower nephron number and higher body mass index, which further highlights the contribution of combined CKD risk factors to glomerular hypertrophy. The findings presented in Chapter 4 suggest that glomerular hypertrophy is a compensatory response to an imbalance between nephron number and body size. However, the main issue with these extrapolations is the lack of longitudinal studies to confirm this hypothesis.
Chapter 5 provides the first report of glomerular number and size in ex vivo human kidneys obtained with magnetic resonance imaging. This represents the first step towards the development of a non-invasive method for quantifying human glomerular number and size in vivo.

Glomerular hypertrophy is often associated with the development of glomerulosclerosis. Recent studies suggest that podocyte depletion is a key step in the development of glomerulosclerosis. Chapter 6 describes the recommended disector/fractionator method for quantifying podocyte number in individual glomeruli when sufficient tissue is available. Chapter 7 shows the application of the disector/fractionator method to estimate podocyte number in glomeruli from children and adults. The results show that glomeruli in children are significantly smaller than in adults, and contain fewer podocytes (452 podocytes per glomerulus compared with 558 in adults). However, while large adult glomeruli contained more podocytes than small adult glomeruli and glomeruli in children, they also presented a significantly lower podocyte density, which may render them more susceptible to glomerulosclerosis. Chapter 8 summarizes the major findings of the thesis and suggests avenues for future research. These include studies of podocyte endowment in children, development of more efficient semi-automated methods for counting podocytes, and studies of podocyte numbers in renal biopsies.
GENERAL DECLARATION

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master’s regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. This thesis includes 5 original papers published in peer-reviewed journals and 1 unpublished manuscript. The core theme of the thesis is **glomerular hypertrophy in subjects without renal disease and its association with multiple clinical risk factors for renal disease and with podocyte depletion**. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Anatomy and Developmental Biology under the supervision of Professor John Bertram (main supervisor). The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. In the case of **Chapters 2, 3, 4, 5, 6 and 7** my contribution to the work involved the following:

<table>
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<tr>
<th>Thesis chapter</th>
<th>Publication title</th>
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<th>Nature and extent of candidate's contribution</th>
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<td>2</td>
<td>Estimating individual glomerular volume in the human kidney: clinical perspectives</td>
<td>Published</td>
<td>Design of experiment, data analysis, and writing of multiple drafts – 80%</td>
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<tr>
<td>3</td>
<td>Hypertension, glomerular hypertrophy and nephrosclerosis: the effect of race</td>
<td>Published</td>
<td>Data analysis and editing of multiple drafts – 20%</td>
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<td>4</td>
<td>Glomerular hypertrophy in subjects with low nephron number: contributions of sex, body size and race</td>
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<td>Design of experiment, data collection, analysis, writing and editing of multiple drafts – 85%</td>
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<td>5</td>
<td>MRI-based glomerular morphology and pathology in whole human kidneys</td>
<td>Published</td>
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<td>6</td>
<td>Design-based stereological methods for estimating numbers of glomerular podocytes</td>
<td>Published</td>
<td>Data collection, analysis, and writing and editing of multiple drafts – 80%</td>
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<td>7</td>
<td>Podocyte number in children and adults: associations with glomerular size and numbers of other resident glomerular cells</td>
<td>Submitted</td>
<td>Design of experiment, data collection, analysis, and writing and editing of multiple drafts – 90%</td>
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I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed:          Date: 21/5/2014
ACKNOWLEDGEMENTS

I dedicate this thesis to a number of people who have helped me during this PhD adventure.

First, and foremost, I would like to thank my wife, Dr. Milagros Wong, for her support and patience, but more importantly for her dedication and love as a partner, which allowed me to devote significant time to my PhD studies.

I would like to acknowledge my family in Peru, Guido Puelles, Carolina Rodriguez and Juan Diego Puelles. Their teachings, advice and love have given me the necessary strength to work hard and give my very best every day of this degree.

I would like to acknowledge the excellent guidance of my three supervisors, Prof. John Bertram, Prof. Wendy Hoy and Prof. Peter Kerr. I have been lucky enough to be mentored by these three Australian luminaries in kidney research. Peter was generous enough to give me his time, advice and clinical perspective in every experiment. He has an open-door policy, always helpful and willing to assist in every way. Wendy was the one that brought me to Australia and believed in me when I had very little to offer. During my PhD, she has always given me her expert opinion, but more importantly, she has always guided me to thrive for research excellence, in every experiment and with every manuscript. Throughout these years, Wendy and Peter have made invaluable contributions to the work presented in this thesis.

John deserves a special mention. Not only my main supervisor, but also an excellent mentor who taught me from day one, not only about science, but also about the life of the researcher. He took me under his wing, and groomed me as a young scientist, but more importantly as a leader. Furthermore, John was always concerned about my health and well-being; always making sure that I was not overwhelmed by my PhD and encouraging me to
maintain a balanced life. John and members of his laboratory, including Dr. Georgina Caruana, Dr. Luise Cullen-McEwen, Ms Rebecca Douglas-Denton and Ms Stacey Hokke were always available for assistance, feedback and troubleshooting. More importantly, this was an excellent group of people to work with, who created a fun environment to perform high quality research. Other members of the Department of Anatomy and Developmental Biology at Monash University, including Dr. Jinhua Li, Dr. James Armitage and Associate Prof. Sharon Ricardo and international collaborators, including Dr. Scott Beeman (Arizona State University, USA), Prof. Kevin Bennett (University of Hawaii, USA) and Prof. Jens Nyengaard (University of Aarhus, Denmark), have also provided significant assistance during my PhD studies.

I would like to extend a well-deserved thank you to Dr. Michael Hughson who collected all the autopsy tissue used in the experiments of this thesis. Furthermore, I would like to thank the families that allowed this collection in Mississippi, USA. These autopsy studies have helped to build the foundation of important principles in current nephrology, none of which could have been possible without the generosity of these families.

Finally, each experiment required particular technical expertise, which were kindly provided by personnel of multiple core facilities: Monash Micro Imaging (Mr Chad Johnson, Mr Stephen Firth and Dr. Alex Fulchner), Monash Histology Platform (Ms Camilla Cohen, Ms Stephanie Tombs, Mr Jonathan Bensley, Ms Julie Hickey and Ms Sue Connell), and Ms Susan Mott from the Centre of Chronic Disease at the University of Queensland.
Manuscripts


**Abstracts**

- **Puelles VG**, McNamara B, Li J, Salih F, Hughson MD, Hoy WE, and Bertram JF. Podocyte number per glomerulus varies within and between human kidneys; 8th International Podocyte Congress, Bristol, UK, 2010. **Poster presentation.**


- **Puelles VG**, Douglas-Denton RN, Hughson MD, Hoy WE, Nyengaard JR and Bertram JF. A design-based method for estimating total podocyte number in individual renal glomeruli; 13th International Congress of Stereology, Beijing, China, 2011. **Oral presentation.**


• **Puelles VG**, Douglas-Denton RN, Zimanyi MA, Hughson MD, Hoy WE, and Bertram JF. Glomerular number and individual glomerular volume in female Americans; American Society of Nephrology (ASN) Kidney Week, San Diego, USA, 2012. **Poster presentation.**

• **Puelles VG**, Douglas-Denton RN, Hughson MD, Hoy WE, Nyengaard JR and Bertram JF. Relative podocyte depletion in obese Caucasian American men without renal disease; ASN Kidney Week, San Diego, USA, 2012. **Poster presentation.**

• **Puelles VG**, Hughson MD, Hoy WE, and Bertram JF. Numbers of podocytes and parietal epithelial cells in the normal human kidney: associations with glomerular size. 49th Annual Scientific Meeting of the Australian and New Zealand Society of Nephrology, Brisbane, QLD Australia, (37-37). 9-11 September 2013. **Oral presentation.**

• **Puelles VG**, Hughson MD, Hoy WE, and Bertram JF. Numbers of parietal epithelial cells in the normal human kidney: associations with podocyte number and glomerular size. International Society of Nephrology Forefronts Meeting: Stem Cells and the Kidney, Florence, Italy, 2013. **Poster presentation.**

• **Puelles VG**, Cullen-McEwen LA, Hughson MD, Hoy WE, and Bertram JF. Podocyte number in the normal human kidney: are all glomeruli the same? 10th International Podocyte Conference, Freiburg, Germany, 2014. **Poster presentation.**

• **Puelles VG**, Cullen-McEwen LA, Hughson MD, Hoy WE, and Bertram JF. Podocyte number in children and adults: associations with glomerular hypertrophy; 10th International Podocyte Conference, Freiburg, Germany, 2014. **Poster presentation.**
ABBREVIATIONS

APOL1: Apo-Lipoprotein 1
BC: Bowman’s capsule
BMI: body mass index
BSA: body surface area
CD2AP: CD2-associated protein
CF: cortical fibrosis
CKD: chronic kidney disease
DAPI: 4’,6-diamidino-2-phenylindole
ECM: extracellular matrix
eNOS: endothelial nitric oxide synthase
ESKD: end stage kidney disease
FSGS: focal and segmental glomerulosclerosis
GBM: glomerular basement membrane
GS: glomerulosclerosis index
HIVAN: human immunodeficiency virus associated nephropathy
HTN: hypertension
IGBC: integrative glomerular barrier complex
IGV: individual glomerular volume
ITR: intimal thickness ratio

JGA: juxtaglomerular apparatus
K/DOQI: Kidney Disease Outcomes Quality Initiative
LBW: low birth weight
MCD: minimal change disease
MRI: magnetic resonance imaging
Neph: nephrin
N_{glom}: total nephron number
PDGF: platelet derived growth factor
PDX: podocalyxin
PECs: parietal epithelial cells
RTT: renal replacement therapy
SNP: synaptopodin
T1D: type 1 diabetes
T2D: type 2 diabetes
V_{glom}: mean glomerular volume
vWF: von Willebrand factor
WT-1: Wilms’ Tumour 1
Chapter 1

Introduction
1.1 Chronic kidney disease (CKD)

1.1.1 Overview

1.1.1.1 Definition and stages

CKD is often described as a “silent killer” due to its insidious latency period at early stages followed by the late onset of signs and symptoms as a result of complications of reduced renal function [1]. Although early detection is feasible and beneficial, it frequently represents a major clinical challenge [2, 3].

The diagnosis of CKD is based on three assessments: renal function (i.e. glomerular filtration rate, GFR), indirect markers of renal damage (i.e. albuminuria) or direct evidence of renal pathology (i.e. renal biopsy) [4]. The first two of these are the least invasive and most cost-effective [5]. However, both are problematic with regards to reliability and validity, and there is an ongoing debate as to the best measurement methods [6-8].

Guidelines for CKD definition and classification were provided by the Kidney Disease Outcomes Quality Initiative (K/DOQI) [9] and were based on duration of signs and symptoms (at least 3 months), GFR (ml/min/1.73m² of body surface area) and/or the presence of kidney damage (i.e. albuminuria). The overall definition of CKD and details of GFR-based CKD stages are detailed in Table 1. These stages range from normal renal function (stage 1; GFR > 90 ml/min per 1.73m²), where renal injury could be present (i.e. albuminuria) but renal function has not been altered, to renal failure or end stage kidney disease (ESKD) (stage 5; GFR < 15 ml/min per 1.73m²), which requires renal replacement therapy (RRT) – dialysis or transplantation as life-sparing interventions. While transplantation usually represents a “cure”, dialysis and other supportive measures come at a high burden for all involved parties, including patients, families, health services and governments [10].
**Chronic Kidney Disease**

<table>
<thead>
<tr>
<th>Definition</th>
<th>Functional criteria</th>
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<tbody>
<tr>
<td></td>
<td>GFR &lt; 60 mL/min per 1.73m² for &gt; 3 months</td>
</tr>
<tr>
<td></td>
<td>Structural criteria</td>
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<td></td>
<td>Kidney damage for &gt; 3 months (i.e. albuminuria)</td>
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<table>
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<tr>
<th>Staging</th>
<th>GFR-based categories (mL/min per 1.73m²)</th>
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<tbody>
<tr>
<td></td>
<td>Stage 1 ≥ 90 (normal or high)</td>
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<td></td>
<td>Stage 2: 60-89 (mildly decreased)</td>
</tr>
<tr>
<td></td>
<td>Stage 3a: 45-59 (mildly/moderately decreased)</td>
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<tr>
<td></td>
<td>Stage 3b: 30-44 (moderately to severely decreased)</td>
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<td></td>
<td>Stage 4: 15-29 (severely decreased)</td>
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<td></td>
<td>Stage 5: &lt;15 (renal failure/ESKD)</td>
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</table>

*Table 1:* Chronic kidney disease definition and stages. GFR: glomerular filtration rate. Adapted from [11]

### 1.1.1.2 Epidemiology

CKD is a global pandemic with about 10% of the worldwide population diagnosed with CKD and about 1.6 million people living with ESKD worldwide [12, 13]. CKD affects nearly 26 million adults in the United States of America (USA), which is about 13% of the adult population. With an average survival between 3 and 5 years in the USA, the prevalence of ESKD is nearing 1800 cases per million [14]. Moreover, the incidence of ESKD is as high as 400 cases per million in the USA, Taiwan and some regions of Mexico [15].

According to the Australian Institute of Health and Welfare (2010) annual report [16], CKD affects 16% of Australian adults and the number of new cases of ESKD increased by 167% between 1989 and 2009. This same report provided evidence of a continuous and considerable increase in the number of incident cases of treated ESKD in Australia over the past two decades, an increase of 202% for males and 128% for females with 30% population growth.

### 1.1.1.3 Cost and burden

Many developed nations spend more than 2–3% of their annual healthcare budget on ESKD, while ESKD patients represent only 0.02–0.03% of the total population [17]. Despite the fact
that costs of ESKD appear overwhelming under the Medicare system in the USA, the economic burden associated with mild stages of CKD accounts for more than twice the total cost of ESKD and expenditure on CKD patients exceeded $60 billion in 2007 versus $25 billion for ESKD [18]. In 2011, total US Medicare costs rose 5% to $549 billion, while costs for ESKD reached $34.3 billion, accounting for 6.3% of the Medicare budget, an increase of 5.4% from 2010 [19].

Low renal function and albuminuria have both been independently associated with an increased risk of mortality and ESKD, equally across three major races, Asians, Caucasians, and African Americans despite differences in demographic and clinical characteristics [20]. At a personal level, CKD also has a powerful social impact, being associated with reduced quality of life, decreased productivity and job losses, family pressures and mental disorders [21, 22]. Therefore, the understanding of CKD risk factors is essential for the development of effective preventive strategies.

1.1.2 Risk factors

The 2010 US Renal Data System Annual Data Report identified diabetes (153 per million population), hypertension (99 per million population), and glomerulonephritis (23.7 per million population) as the three leading causes of renal failure [18]. This is consistent with the increase in the burden of modifiable risk factors for CKD, such as obesity, diabetes and hypertension [14], not to mention non-modifiable factors that are not fully understood, such as older age and male sex.
1.1.2.1 Type 2 diabetes (T2D) and hypertension

Over the past two decades, the prevalence of T2D has nearly doubled in the USA, and has increased between 3 and 5-fold in India, Indonesia, China, Korea and Thailand [23]. According to the World Health Organization, China and India will have about 130 million people with diabetes in 2025, and it is expected that these patients will consume about 40% of total health expenditure in those two countries [24]. Furthermore, the global cost of dialysis is predicted to be US$ 1.1 trillion for diabetic nephropathy in 2025, now the most common cause of ESKD worldwide accounting for 30% of the burden [23].

In the USA, more than 5% of people with newly diagnosed T2D already have CKD, and an estimated 40% of both type 1 and type 2 diabetics will develop CKD during their lifetimes; the majority within 10 years of diagnosis [25]. Moreover, CKD in diabetes carries an increased risk of progression to ESKD, with almost half of patients with ESKD in most developed countries having diabetes [23]. In Australia, the Institute of Health and Welfare (2010) predicted that diabetes is likely to account for 64% of new cases of treated ESKD in 2020, a 20% increase compared to the current frequency [16].

Nearly 1 billion people worldwide have high blood pressure. By 2025 the number of hypertensive patients is expected to increase to 1.56 billion, with an increase of 24% in developed countries and 80% in developing regions [26]. The relationship between hypertension and kidney disease is a complex and apparently synergistic one. While kidney dysfunction is a major cause of hypertension, hypertension in turn aggravates CKD and accelerates its progression [27]. Moreover, the renal consequences of hypertension are uniquely exaggerated in the CKD population due to loss of the normal nocturnal decline in blood pressure [28]. These topics are extensively reviewed by Najafian et al. [29], Ritz et al. [30] and Hill [31].
1.1.2.2 Obesity

Obesity is a worldwide epidemic [32] that usually co-exists with T2D and hypertension [33]. However, a 2008 meta-analysis by Wang et al. [34] provided evidence of obesity being an independent risk factor for CKD. Furthermore, among 75,000 adults followed over 21 years, increased body mass index (BMI) dramatically correlated with initiation of RRT or death with CKD, highlighting the critical role of obesity in disease progression and outcome [35].

A meta-analysis by Thomas et al. [36] showed that metabolic syndrome and its components (including central obesity, dyslipidaemia, elevated blood pressure and impaired fasting glucose levels) are associated with the development of CKD and markers of kidney damage. A more recent study found evidence of hyperfiltration in obese adults with higher GFR (61%), renal plasma flow (28%) and filtration fraction (27%) than normal-weight controls [37]. Furthermore, secondary glomerular hypertrophy appears to play a pivotal role in these pathological changes [38-43].

In 1974, an association between morbid obesity and renal pathology was first described [44]. In 2001, Kambham et al. [39] reported the first large renal biopsy-based study on obesity-related glomerulopathy, a variant of idiopathic focal and segmental glomerulosclerosis (FSGS). Obesity-related renal disease presented a lower incidence of nephrotic syndrome, consistent presence of glomerular hypertrophy, fewer segmental lesions and milder podocyte foot process effacement than idiopathic FSGS [39]. Chen et al. [38] provided evidence of this emerging problem in China and also highlighted a feature of concern: although marked glomerular alterations could be observed in renal pathology (including podocyte injury and glomerular hypertrophy), proteinuria was mild to moderate, which potentially masks the diagnosis and thereby early detection of disease. A similar pattern was described by Serra et al. [40], who found that among extremely obese patients with normal
renal function, the majority showed renal histopathology, but only 4% had macroalbuminuria (≥300 mg/day) and 41% presented with microalbuminuria (30-300 mg/day). Because only macroalbuminuria is detectable with urinary dipstick, the main tool used for CKD screening [45], 96% of these patients will go undetected with a screening workup, which highlights major difficulties with early disease detection.

Although there are multiple mechanisms that predispose obese individuals to kidney disease, evidence suggests that glomerular size (see Section 1.3) and podocyte injury (see Section 1.4.2) may play key roles and should be considered as targets for early detection and progression of obesity-related kidney pathology.

1.1.2.3 Sex

In patients with CKD, the prevalence of renal failure is higher in males than in females [46]. Neugarten et al. [47] provided the largest meta-analysis on sex differences in CKD to date. Their analysis of 11,345 patients in 68 studies showed that females with several glomerulopathies (such as polycystic kidney disease and membranous glomerulonephritis) had a more favourable renal outcome than males. Although the incidence of systemic lupus is higher in females, the frequency and severity of renal involvement is greater in males [48]. A similar pattern is observed in type 1 diabetes (T1D), with males presenting an increased risk for the development of early diabetic nephropathy [49]. Furthermore, outcome and survival of renal grafts was found to be better in female than male recipients [50], suggesting that the female “environment” may be protective of the transplanted graft.

These findings in humans are paralleled in many animal models of renal disease, such as aging, renal ablation, hypertension, and polycystic kidney disease, in which females are relatively protected from the development of glomerulosclerosis and other patterns of renal
disease compared to males [51]. In both humans and rodents, the specific causes underlying the sex differences in renal disease progression are unclear but have been speculated to involve multiple factors including kidney size, body size, nephron number, systemic/glomerular haemodynamic changes, diet, and the direct effects of oestrogens and androgens on renal tissue [51].

1.1.2.4 Race

Evidence from epidemiological studies supports the conclusion that certain racial groups have a higher risk of developing CKD than other groups. Populations at higher risk include Australian Aborigines [52, 53] and African Americans [54-56]. While this predisposition to CKD is clearly multifactorial, these factors appear to differ between the racial groups.

Many Australian Aborigines have lived for decades under precarious conditions, with poverty and scarce access to health care. This challenging socio-economic environment combined with a radical and rapid change in culture (i.e. westernization) has placed this population at great risk [57, 58]. Furthermore, Aboriginal mothers often live under poor socio-economic conditions, which impacts adversely on the feto-maternal environment during pregnancy as evidenced by high rates of low birth weight offspring [59].

Low birth weight (LBW), defined as a term birth less than 2.5 Kg, has been closely correlated with low numbers of nephrons [60], which in turn is associated with glomerular hypertrophy. In a small study, Aborigines were found to have approximately 20% fewer nephrons than Australian whites from the same geographic region, and glomerular hypertrophy has been a ubiquitous finding in Aborigines in both autopsy and biopsy specimens [61].
There is clear epidemiological evidence that African Americans have a greater frequency of hypertension and in turn cardiovascular-related deaths (i.e. strokes) than Caucasian Americans, but the cause of this disparity remains unclear. African Americans also have higher rates of CKD and ESKD than American whites [54]. The kidneys of African Americans have similar numbers of nephrons as whites [62, 63], and Zimanyi et al. [64] showed that unlike Caucasian Americans, African American males were not “protected” from glomerular hypertrophy by high nephron number. The excessive glomerular hypertrophy observed in African Americans may be explained by high rates of cardiovascular disease (i.e. essential hypertension) and specific genetic variants [63-66].

Among African Americans, a particular gene that deserves special attention is located on chromosome 22. It encodes Apo-Lipo-protein 1 (APOL1) and recent studies have revolutionized the principles of genetic-associated risk for CKD [67-70]. Mutations of the APOL1 gene are highly prevalent among populations of African descent [71, 72] and seem to be an evolutionary protective factor against trypanosome parasitic infection [67]. However, the presence of these mutations in African Americans is closely associated with very large relative risks for CKD, including a 5-17-fold increase in risk for FSGS, and a 29-fold increase in risk for human immunodeficiency virus associated nephropathy (HIVAN) [69, 70].

1.1.2.5 Nephron number and birth weight

Variability in nephron number within species of wild-type experimental animals tends to be quite small with a typical coefficient of variation (standard deviation as a percentage of the mean) of about 10% [73]. This is most likely due to the fact that experimental animals are maintained under standardized housing conditions, are fed controlled diets and are often inbred. However, there is increasing evidence from animal studies that a range of
perturbations to the feto-maternal environment result in a nephron deficit that is associated with cardiovascular and renal physiological abnormalities, as originally proposed by Brenner et al. [74] more than 25 years ago. These perturbations include nutrient restriction, low protein diet, and maternal exposure to natural and synthetic glucocorticoids, alcohol and certain antibiotics [75].

In contrast to experimental animals, nephron number varies widely in normal human kidneys. The Monash Series, which includes kidneys collected at autopsy from more than 420 participants from five different racial groups (white and Aboriginal Australians, Caucasian and African Americans, Senegalese), reported a 13-fold range in nephron number, from 0.21 to 2.70 million nephrons per kidney [63]. This great variability in nephron number in subjects without overt renal disease highlights the resilience and adaptability of the human kidney, even in a setting of apparent extreme nephron deficit.

Importantly, human nephron number is closely associated with birth weight [76, 77]. In a study of Caucasian and African Americans, Hughson et al. [76] reported an additional 257,426 nephrons per kidney per kilogram increase in birth weight. The first report of a link between LBW and kidney disease was observed in Australian Aborigines [59, 78]. Subsequent reports have described relationships between LBW and reduced renal function in young Norwegian adults [79], microalbuminuria and abnormal renal function in Dutch adolescents [80], and ESKD in American [81] and Norwegian adults [82]. In a recent meta-analysis, the combined odds ratio for risk of albuminuria showed that people with albuminuria were 81% more likely to have a history of LBW and 58% more likely to develop ESKD [83]. Hodgin et al. [84] reported an association between FSGS and LBW, suggesting that low nephron endowment independently promotes the development of secondary FSGS. Taken together, people with surrogate markers of low nephron number, such as LBW, may
be at a greater risk of developing renal disease later in life. Further investigations in longitudinal human cohorts are urgently needed.

It is worth noting that while CKD risk factors have been considered in this Chapter as independent variables, patients coming to the clinic do not typically present with isolated risk factors. Rather, patients typically present with multiple conditions, highlighting the multifactorial nature of the aetiology of CKD.

1.1.3 Treatment versus prevention

As described above, established CKD can progress to ESKD, at which stage patients require RRT in order to survive. While renal transplantation is often considered a “cure”, some diseases that lead to CKD and ESKD can recur after transplantation. FSGS is a well-known example of this phenomenon [85-87]. While renal dialysis is among the most effective, life-saving clinical interventions ever developed [88], it is not sufficient to fully restore full health in most patients with ESKD.

A conceptual model of CKD depicted in Figure 1 describes the current understanding of CKD as a complex entity with an intricate progression pattern. The conceptual model of CKD was first presented by K/DOQI in 2002 [89], as part of a public health initiative for the prevention of the development, progression and complications of CKD [90]. This model identifies ESKD and links it to all the earlier stages of CKD. This cascade of events begins with kidney damage, which progressively leads to a reduction in GFR and eventually ESKD.

Furthermore, the K/DOQI model directs the focus to the identification of populations at high risk of developing CKD based on the presence of risk factors, which is the main focus of this thesis. While some of these risk factors may be modifiable (including obesity and T2D), others are not (such as sex and advanced age). However, in most cases, interventions at
earlier stages may slow or prevent the progression of renal disease [90]. It is in this pre-disease setting where glomerular hypertrophy is first observed, and for this reason glomerular hypertrophy is a major research focus of this thesis. The following sections discuss glomerular injury and glomerular hypertrophy in the context of their contributions to CKD and pre-disease stages.

**Figure 1: The conceptual model of CKD.** Horizontal arrows show transitions between stages (outcomes). Solid arrows pointing from left to right show progression of kidney disease. Dashed arrowheads pointing from right to left show disease remission. Grey triangles show the continuous nature of changes in glomerular filtration rate and markers of kidney damage. Adapted from [11].

### 1.2 Glomerular injury

Approximately 90% of kidney diseases leading to ESKD originate in glomeruli [19]. Diabetic nephropathy, the number one cause of CKD and ESKD, features significant glomerular pathology [29], as do highly prevalent adult pathologies such as IgA nephropathy [91],
primary FSGS [92] and membranous nephropathy [93]. The research presented in this thesis focuses on glomerular morphological features, primarily glomerular volume and podocyte number, and their associations with CKD risk factors.

1.2.1 Glomerular function

Each nephron consists of a glomerulus (for filtration of blood) and an associated tubule with several differentiated segments (for modification of the filtrate). The kidneys are perfused by 20% of the cardiac output, form approximately 180L of ultrafiltrate per day, and produce and excrete approximately 1.5L of urine daily. While the tubular component of the nephron plays a major role in the production of urine, the glomerulus (and thereby the glomerular filtration barrier) is the first step for the generation of the ultrafiltrate.

1.2.2 The glomerular filtration barrier

The glomerular filtration barrier consists of three specialized layers: (1) fenestrated endothelial cells that form a dense network of anastomosing capillaries; (2) the negatively charged glomerular basement membrane (GBM) which is composed mainly of type IV collagen, heparan sulphate proteoglycans, laminin and fibronectin; and (3) visceral epithelial cells called podocytes (Figure 2A).

The glomerular filtration barrier is permeable to water, small solutes and low-molecular-weight proteins up to the mass of albumin (approximately 65kDa), but largely prevents the passage of plasma proteins larger than 70 kDa, especially if they are negatively charged [94]. Alterations to the chemical or structural integrity of the filtration barrier can result in an alteration in GFR or increased leakage of proteins into the urinary space (and ultimately
urine), both of which are classic signs of renal disease [95]. Furthermore, the integrative glomerular barrier complex (IGBC) (Figure 2B) is composed of the glomerular endothelial surface layer, endothelial cells, the GBM, and podocytes with their foot processes and slit membranes; and it is also directly affected by mesangial cells.

Figure 2: The glomerular filtration barrier. (A) Scanning electron micrograph of a portion of a glomerulus showing podocyte cell bodies (CB), major processes (MP), secondary processes (SP) and foot processes (FP). Dashed box represents the focus area for the integrative glomerular barrier complex (IGBC). (B) The IGBC is composed of the glomerular endothelial surface layer (ESL), endothelial cells, the glomerular basement membrane (GBM), and the podocyte, with its foot processes and their slit membranes, but is also affected by mesangial cells. All of these structures secrete and are direct targets of multiple molecules that maintain homeostasis and allow it to fulfill its filtration function. Ang: angiopoietin; ANGPTL: angiopoietin-like protein; EGF: epidermal growth factor; ET-1: endothelin-1; HGF: hepatocyte growth factor; IGFBP-rP1: insulin-like growth factor binding related protein 1; IL-1: interleukin-1; MMP-9: matrix metalloproteinase-9; NO: nitric oxide; PDGF: platelet-derived growth factor; sFLT-1: soluble fms-like tyrosine kinase-1; TNF-α: tumour necrosis factor-α; VEGF: vascular endothelial growth factor. Adapted from [96, 97].

In normal conditions, the IGBC operates in perfect homeostasis in order to fulfill its filtration function. For example, podocytes produce factors that affect the properties of glomerular endothelial cells, including angiopoietins, angiopoietin-like protein 3, angiopoietin-like protein 4, insulin-like growth factor binding protein-related protein 1, and endothelin-1. In turn, endothelial cells secrete factors required for optimal podocyte and mesangial cell function, including epidermal, insulin-like, hepatocyte, and platelet-derived growth factors.
that may affect both mesangial cells and podocytes. Furthermore, mesangial cells can affect podocytes by the secretion of multiple factors, which include midkine, transforming growth factor β1, nitric oxide, cytokines and chemokines. All of these cell types participate in this intricate and complex signalling network in order to maintain glomerular structural homeostasis [97].

**1.2.3 Resident cells of the glomerulus**

As mentioned in Section 1.2.2, the normal glomerular tuft contains three resident cell types - endothelial cells, mesangial cells and podocytes. Parietal epithelial cells (PECs) form the simple squamous epithelium of Bowman’s capsule, and have been the focus of renewed research interest in recent years following reports that they may contribute to podocyte replacement [98-100] and the development of glomerulosclerosis [101, 102]. A schematic diagram of basic glomerular histology is provided in Figure 3, and each of the four cell types is considered in greater detail below.
1.2.3.1 Glomerular endothelial cells

Endothelial cells are the first component of the glomerular filtration barrier (Figure 4). They have a negatively charged gelatinous surface coat, also known as the glycocalyx, which is composed principally of proteoglycans and sialoproteins [104-110]. Biophysical models indicate that the glycocalyx contributes 50% of the overall hydraulic resistance of the glomerular filtration barrier [94]. Most of the membranous organelles of endothelial cells are located close to the nucleus, in the so-called axial region. From this region, a thin sheet
of cytoplasm extends to surround the capillary lumen [104, 106, 111]. This thin sheet of cytoplasm contains numerous holes or fenestrations with a diameter of 70-100 nm [112]. Unlike fenestrations in most capillaries, those in glomerular capillaries do not contain diaphragms. Fenestrations constitute approximately 20–50% of the glomerular capillary wall surface area [113] and thus provide a large surface area for filtration. GFR is proportional to the total area of fenestrations, and disorders of fenestrations play a role in proteinuria and loss of renal function [114].

Endothelial cell dysfunction plays an important role in the development of proteinuria and the progression of kidney disease. Loss of the glycocalyx is associated with kidney disease with systemic vascular dysfunction [115, 116]. Endothelial nitric oxide synthase (eNOS) knockout mice are more susceptible to glomerulosclerosis [117], even under the highly sclerosis-resistant B6-C57 background [118]. Furthermore, there is significant evidence suggesting that glomerular endothelial dysfunction and damage is a critical contributor to podocyte injury [119-121]. Importantly, endothelial cells have a high proliferative capacity, which allows them to directly contribute to glomerular growth, repair and hypertrophy [111].
Figure 4: Morphological features of glomerular endothelial cells. (A) Schematic shows the spatial location of endothelial cells (capillary endothelium) in the glomerulus. (B) Scanning electron micrograph shows endothelial cell fenestrations. (C) Transmission electron micrograph highlights endothelial fenestrations located in the endothelial cell cytoplasm (arrowheads). Adapted from [112, 122, 123].

1.2.3.2 Mesangial cells

Mesangial cells constitute the central stalk of the glomerulus and are located in the glomerular mesangium (Figure 3). Mesangial cells synthesise and secrete components of the mesangial matrix including types IV and V collagen, laminin (A, B1, and B2), fibronectin, heparan sulphate and chondroitin sulphate proteoglycans, entactin, nidogen, decorin and biglycan [124]. Mesangial cells are contractile, and via anchoring filaments connected to the
GBM, regulate glomerular haemodynamics [125]. Furthermore, in the juxtaglomerular apparatus, gap junctions physically link mesangial cells, vascular smooth muscle cells, endothelial cells, and renin-secreting cells, allowing them to function as a unified system, regulating pre-glomerular vascular tone and renin secretion [126].

Disorders of mesangial cells and the mesangial matrix are observed in numerous settings. For example, mesangioproliferative diseases are typically associated with mesangial immunoglobulin deposition [127]. These diseases include primary diseases such as IgA nephropathy, mesangioproliferative glomerulonephritis and systemic diseases such as lupus nephritis [124]. Moreover, mesangial matrix expansion with diffuse or nodular glomerulosclerosis is a typical feature of diabetic nephropathy [128]. Alterations of mesangial cell biology are also seen in other forms of glomerular damage such as amyloidosis and light-chain deposition disease [129].

Mesangial cells are key players in the development of inflammatory glomerular diseases, based on the deposition of immune complexes combined with complement activation and generation of mediators of inflammation, such as reactive oxygen species, platelet activating factor, cytokines (such as tumour necrosis factor α), and multiple chemokines [127]. The release of these pro-inflammatory mediators can lead to increases in endothelial permeability, allowing more macromolecules to reach the mesangium and further enhance the generation of inflammatory mediators. This increase in inflammatory mediators results in local leukocyte adhesion, activation and extravasation. In turn, the inflammatory cascade alters glomerular permeability by influencing podocyte function, leading to proteinuria and progressive renal disease [124].
1.2.3.3 Podocytes

Podocytes form the outer layer of the glomerular filtration barrier and consist of a cell body, large cytoplasmic processes and smaller foot processes or pedicels (Figure 5A).

Figure 5: Morphological features of glomerular podocytes. (A) Scanning electron micrograph shows main podocyte features, including: CB: cell body, MP: major processes, and FP: foot processes. (B) Transmission electron micrograph showing podocyte foot processes and slit-diaphragms (SD) and their relation to the capillary lumen and basement membrane (BM). Adapted from [130].

The cell body contains the nucleus as well as most of the membranous organelles, including multiple mitochondria, lysosomes, rough endoplasmic reticulum, and numerous Golgi complexes. Podocytes contain an extensive cytoskeletal network, with major processes rich in microtubules and foot processes containing abundant actin microfilaments [131]. The foot processes from adjacent podocytes interdigitate, and filtration slit diaphragms are found between the foot processes (Figure 5B). The slit diaphragm constitutes the final barrier to urinary protein loss [132]. It is a multiprotein complex signal transduction unit with characteristics of a modified adherens junction that spans the 30–50 nm wide filtration slits [106, 133, 134]. A number of slit diaphragm proteins have been identified including ZO-1, FAT-1, Neph1, 2 and 3, P-cadherin and CD2-associated protein (CD2AP) [131, 135].
In addition to their function as a component of the glomerular filtration barrier, podocytes have important endocrine and paracrine roles. Vascular endothelial growth factor (VEGF)-A is a major angiogenic glycoprotein and is highly expressed by podocytes [136]. VEGF receptors are expressed by adjacent glomerular endothelial cells, suggesting a direct effect of VEGF on endothelial cells. In support of this, glomerular endothelial cell injury is observed in a knockout mouse model of selective deletion of one VEGF-A allele from podocytes [137]. The same study demonstrated that tight regulation of VEGF-A expression is necessary for glomerular homeostasis: the glomerular filtration barrier failed to form following deletion of both VEGF-A alleles from podocytes, whereas overexpression of VEGF-A resulted in a collapsing glomerulopathy, the classic renal lesion seen in HIVAN. Podocytes also synthesise and secrete a range of other growth factors including angiopoietins, angiopoietin-like proteins 3 and 4, insulin-like growth factor binding protein-related protein 1, and endothelin-1 [94, 97].

Podocytes synthesise the majority of components of the GBM, including collagen type IV, proteoglycans and glycoproteins [138]. Unlike glomerular endothelial cells and mesangial cells, podocytes have a very limited capacity for replication in the healthy glomerulus. Consequently, podocyte injury and loss are key hallmarks of glomerular disease. Podocytes are a major focus of the studies described in this thesis and are considered in greater detail in Section 1.4.

1.2.3.4 Parietal epithelial cells (PECs)

Parietal epithelial cells (PECs) form the simple squamous epithelium of Bowman’s capsule. While strictly not considered a resident cell of the glomerulus, they are a component of the
renal corpuscle (Figure 3). PECs prevent leakage of the glomerular ultrafiltrate from the urinary space into the peri-glomerular interstitium [139].

PECs have been the subject of much research attention in recent years due to evidence suggesting their potential role as podocyte progenitor cells [98-100] and their direct role in the development of glomerulosclerosis [101, 102]. The first evidence of their possible role in podocyte regeneration or replacement came from Sagrinati et al. [140] who described three subpopulations of PECs based on their expression of specific stem cell markers CD24 and CD133, as well as the podocyte marker podocalyxin (PDX). These subpopulations were defined as: multipotent PECs (CD133+CD24+PDX-), podocyte progenitors (CD133+CD24+PDX+) and parietal podocytes (CD133-CD24-PDX+). A subsequent study from the same group [141] showed that injection of multipotent PECs into mice with glomerular disease leads to renal function recovery and remission of proteinuria, suggesting podocyte replacement. Furthermore, a study by Appel et al. [142] using a transgenic mouse to unambiguously trace PECs showed that PECs migrate from Bowmans’ capsule to the glomerular tuft, and once on the tuft, these cells express podocyte markers and are located outside the glomerular capillaries. Based on these findings, the Romagnani group proposed that a subpopulation of PECs are glomerular stem cells that may differentiate into podocytes and migrate to the glomerular tuft as part of a physiological process [100, 143]. These findings have been supported by publications by Peired et al. [144], Zhang et al. [145-147], and Pichaiwong et al. [148].

However, there is currently great controversy about the possible role of PECs in podocyte replacement [149]. Two recent publications by Wanner et al. [150] and Berger et al. [151] have challenged the potential role of podocyte replacement via PEC progenitor or stem cells. These two studies in rodents both reported very limited capacity to gain new podocytes
during adult life. Taken together, evidence suggests that PECs may contribute to podocyte gain during glomerular development and possibly during physiologic glomerular growth as occurs in the neonatal period and in early childhood [152]. However, evidence that PECs may contribute to podocyte replacement or accumulation in healthy adult glomeruli remains incomplete.

Current understanding of this important issue of podocyte replacement has been hindered by a lack of accurate methods for counting podocytes and other resident glomerular cells. A major aim of this thesis was to develop an accurate and precise method for counting podocytes and other glomerular cells in human glomeruli of known volume.

In conclusion, it is worth noting that glomerular integrity is required to sustain normal renal function. Importantly, injury to every resident glomerular cell type has been shown to lead directly to renal pathology. As such, it is often assumed that glomerular pathology is irreversible. However, this is also a controversial topic, as described below.

1.3 Glomerular hypertrophy

Glomerular hypertrophy is a commonly used term in renal histopathology. However, this term is not only poorly defined but also not well understood, especially in humans. Autopsy studies have shown that normal average adult glomerular volume varies up to 7-fold between subjects and up to 8-fold within subjects [63]. Increases in human glomerular volume are independently associated with multiple CKD risk factors, and are also found in multiple renal diseases [153]. Importantly, a number of important questions remain unanswered and are discussed below.
1.3.1 The hyperfiltration theory

In 1996, Brenner and colleagues [154] revolutionized the world of nephrology by proposing the hyperfiltration theory, which was based on the imbalance between the finite number of nephrons present in a kidney at the completion of nephrogenesis (around 36 weeks gestation in humans) and the physiological demands of an individual. According to this theory, in a setting of nephron under-endowment, which represents low nephron number, remaining nephrons need to compensate in an attempt to match physiologic demands [154]. One adaptive step in this process is an increase in “usage” of the existing nephrons, also known as hyperfiltration [74]. A subsequent (although sometimes parallel) step is an increase in the glomerular filtration surface area to compensate for an imbalance between the available filtration surface area and the physiologic demands of an individual [155]. This hypothesis has been confirmed in a number of animal models in which glomerular hypertrophy has been shown to occur prior to the development of overt renal pathology [156, 157]. Glomerular hypertrophy is also observed following uninephrectomy, occurs in association with aging and a high-fat diet, and is observed in the early stages of diabetes and FSGS [63].

It is worth noting that while the classical view of glomerular hypertrophy (Figure 6) was based on reductions in renal mass, human autopsy studies have shown that glomerular hypertrophy is independently associated with several CKD risk factors, including older age, obesity, hypertension, lower birth weight and African American race [65, 158].
Figure 6: The classical view of glomerular hypertrophy. White circles represent glomeruli that increased in volume from (1) to (2), as a physiological response in order to increase the filtration surface area.

However, just as the aetiology of CKD is multifactorial, glomerular hypertrophy may also occur due to a range of factors. Both of these topics are the focus of the experimental studies described in Chapters 2, 3 and 4 of this thesis.

1.3.2 Glomerular volume in subjects without renal disease

Most reports of human glomerular volume to date have reported mean values, in other words, a single mean value for an entire kidney. It is well known that glomerular volume in a given human kidney varies through the life course. Human glomeruli have been reported to increase in size approximately 7-fold from infancy to adulthood [159] and then to decrease in size during senescence [76, 160, 161].

Mean glomerular volume varies widely in normal human kidneys. In the Monash Series of 420 kidneys obtained at autopsy, a 7-fold range in mean glomerular volume was reported [63]. While mean glomerular volume is directly correlated with kidney weight and body size, it is inversely correlated with nephron number and birth weight [76, 161]. Nyengaard and
Bendtsen [160] reported significant direct correlations between mean glomerular volume, kidney weight and body surface area (BSA) in their autopsy study of Danish subjects without renal disease. Hughson et al. [76, 162] confirmed these findings in an autopsy study of African and Caucasian Americans. McNamara et al. [163] described a similar pattern in an autopsy study of 28 Senegalese subjects, showing a close relationship between BSA and mean glomerular volume in combined data from children and adults, reinforcing the concept of body growth driving glomerular growth before adulthood.

Mean glomerular volume is a valuable indicator of glomerular size, but provides no insight into glomerular size variability within subjects. Studies of individual glomerular volume (IGV) in autopsy tissue from male Senegalese, African and Caucasian Americans applying the physical dissector/Cavalieri stereological principles have been published since 2005 [64, 65, 158, 164-167]. In these studies, the volumes of 30 individual glomeruli were measured in each kidney – 10 glomeruli from each of the outer, mid and inner cortex. In these studies, CKD risk factors including older age, low nephron number, obesity, lower birth weight, hypertension and African American race were shown to be independent contributors to glomerular hypertrophy [65, 158, 166].

Ethnic differences provide a unique perspective on the regulation of glomerular size [65]. Glomerular hypertrophy is more prevalent among African American kidney donors and predicts worse outcomes after renal transplant [168, 169]. Furthermore, among subjects from African ancestry, African Americans have larger and more variable glomerular size than do Senegalese subjects, which points towards environmental factors affecting glomerular hypertrophy. In contrast, glomeruli from Caucasian Americans tend to be smaller and more homogenous in size than those from African Americans, suggesting the presence of genetic drivers of glomerular volume.
Interestingly, McNamara et al. [66] found that mean glomerular volume was 54% larger in African Americans than in Senegalese, and 38% larger after adjustment for body size. Considering the similar genetic background of these two populations and their almost identical nephron number, this remarkable difference in glomerular volume suggests a major role for environmental factors, including diet and obesity, in determining glomerular hypertrophy.

Based on the discussion above, the studies described in Chapter 3 tested the hypothesis that kidneys of subjects with sufficient nephrons to cope with increased physiologic demands do not demonstrate glomerular hypertrophy, whereas those with a combination of low nephron number and large body size are likely to undergo glomerular hypertrophy in order to sustain normal renal function. A similar hypothesis, testing the combined effects of African American race and other CKD risk factors (age, nephron number, hypertension and obesity) on glomerular size is described in Chapter 4. The findings presented in Chapters 2, 3 and 4 provide evidence supporting the clinical relevance of glomerular hypertrophy. However, estimation of glomerular volume is currently limited to histological studies. Chapter 5 provides the first report of non-invasive estimation of glomerular volumes in ex vivo human kidneys obtained using magnetic resonance imaging (MRI).

1.3.3 Glomerular hypertrophy in subjects with renal disease

Enlarged glomeruli have been identified in a wide range of renal pathologies, including cyanotic heart diseases [170], oligomeganephronia [171, 172], reflux nephropathy [173], IgA nephropathy [174-177], mesangial proliferative glomerulonephritis [178], obesity-related glomerulopathy [38, 39, 179-181], diabetes [182-186] and FSGS [63, 187, 188]. However, it is
hard to determine whether glomerular hypertrophy is a direct component of these pathologies or a secondary response to an established glomerular insult.

Nishimoto et al. [189] provide evidence that glomerular hypertrophy precedes the development of glomerulosclerosis in adult FSGS patients and is reversible when patients are in remission. Furthermore, Young et al. [190] showed that in Australian Aborigines, glomerular size increased with the degree of glomerulosclerosis, but then decreased markedly when glomeruli were completely sclerosed. Similarly, in mesangial proliferative glomerulonephritis, Daimon et al. [178] reported that glomerular size increased initially and thereafter decreased progressively. These observations suggest glomerular hypertrophy is secondary and has a “point of no return”, after which complete loss of function is achieved with the development of global sclerosis and total capillary collapse.

Among Australian Aborigines, glomerular enlargement is notable in individuals from remote regions, but not those living closer to large population centres [191]. This excessive glomerular hypertrophy, also referred to as glomerulomegaly, probably represents compensatory hypertrophy caused by low nephron number, which probably underlies a developmentally programmed susceptibility to renal disease [57]. This conclusion was supported by Hodgin et al. [84] who provided evidence of very LBW and prematurity promoting the development of secondary FSGS with all patients showing clear signs of glomerular hypertrophy. Interestingly, in patients with reduced renal mass (solitary kidney), superimposition of IgA nephropathy may be frequently associated with a progressive course of disease [192]. Furthermore, Kataoka et al. [177] suggested that glomerular volume could be a simple quantitative prognostic indicator of disease progression in patients with IgA nephropathy.
Patients with T1D and microalbuminuria show signs of glomerular hypertrophy associated with the development of glomerulopathy [193]. Furthermore, Osterby et al. [194] showed that in T1D patients, glomerular volume was significantly higher than in controls and was closely associated with mesangial expansion. In contrast, Bertani et al. [195] showed that T2D patients showed glomerular hypertrophy, mesangial sclerosis and arteriolar hyalinosis. While Fioretto et al. [196] showed that recipients of pancreas transplants (and thereby achievement of glycaemic control) showed smaller glomerular volumes than matched controls, Mauer et al. [197] reported that early blockade of the renin-angiotensin system in patients with T1D did not slow the progression of nephropathy.

The limit between physiological and pathological glomerular hypertrophy remains undefined. Given that in the majority of cases glomerular hypertrophy is associated with an increase in filtration surface area, it is important to consider the effect of an enlarged filtration surface area on glomerular resident cells. Perhaps the cell at greatest risk in the hypertrophied glomerulus is the podocyte, given its very limited capacity to replicate.

1.4 Podocytes

Podocytes are key cells in the context of glomerular structure and function, not only due to their critical structural role, but also because of their high metabolic activity and paracrine functions [198, 199]. Details of podocyte structure and their synthetic and paracrine roles were provided in Section 1.2.3.3. Below, aspects of podocyte development and proliferation are considered, both of which are central to the question of podocyte endowment and replacement/replication in adulthood, and thereby to podocyte depletion. In addition, findings from previous studies of podocyte number in human glomeruli are specified.
1.4.1 Podocyte development and replication

During nephrogenesis, epithelial precursors give rise to mature podocytes. Glomerular development proceeds in four stages: the renal vesicle stage, the S-shaped body stage, the capillary loop stage, and the maturing-glomeruli stage [200, 201]. During the early developmental stages, simple undifferentiated epithelial cells in the lower limb of the S-shaped body form the immature podocyte precursor cell population [202]. At this stage, expression of the podocyte-specific marker podocalyxin (PDX) [203] and the tight junction protein ZO-1 [204] begins, and expression of the podocyte-specific transcription factor Wilms’ Tumour 1 (WT-1) is at its peak [205]. As these immature podocytes enter the capillary loop stage, they lose their mitotic activity and begin to establish their characteristic complex cell architecture, including the appearance of foot processes and the reorganization of cell-cell junctions into slit diaphragms (see Section 1.2.3.3) [202]. Once full maturity is reached, podocytes express cyclin-dependent kinase inhibitors such as p16\(^{INK4a}\), p21\(^{Cip1}\), p27\(^{Kip1}\) and p57\(^{Kip2}\), which negatively regulate cell mitosis, inducing senescence [206]. After this point, podocytes are no longer able to undergo mitosis. Nadasdy et al. [207] studied the expression of different markers of cellular replication such as proliferating cell nuclear antigen and Ki-67 in normal human kidneys, and found that podocytes had the lowest replication capacity of all glomerular cell types.

Cell division requires a rearrangement of the podocyte actin cytoskeleton, which would disrupt the podocyte foot processes, thereby limiting their capacity to divide [208-210]. Whilst podocytes are generally believed to be terminally differentiated and incapable of undergoing mitosis in the normal adult kidney, podocyte proliferation occurs under certain pathological conditions such as collapsing glomerulopathies, which includes HIVAN [211]. These podocytes express Ki-67, lose WT-1 expression and are often bi-nucleated, which
suggests they can replicate their DNA but cannot undergo cytokinesis [212]. Interestingly, podocytes can be induced to proliferate in vitro when cultured from freshly isolated glomeruli [213]. However, cultured podocytes only express low-levels of many of the podocyte-specific differentiation markers, suggesting they only proliferate once they have de-differentiated. Therefore, podocyte mitosis in vivo in adulthood is generally accepted as a catastrophic event leading to further podocyte loss and glomerulosclerosis [214].

1.4.2 Definition of podocyte depletion

The central role of podocyte loss and injury leading to the development of glomerulosclerosis was first described in a rat model of uninephrectomy. In this study, Nagata et al. [215] showed that in a setting of compensatory glomerular hypertrophy, podocytes undergo hypertrophy in order to cover the enlarged capillary area, which leads to podocyte loss, areas of denuded GBM and development of adhesions: the main pathological steps for the development of segmental glomerulosclerosis. As mentioned in Section 1.4.1 podocytes cannot re-enter the cell cycle, and therefore lost podocytes cannot be replaced by mitosis [214], with evidence suggesting that podocyte injury promotes further podocyte injury, leading to a vicious cycle of glomerulosclerosis [208, 216, 217]. This principle has led to the well-accepted dogma that lost podocytes cannot be replaced by cell division, and has turned the attention to other possible sources of podocyte gain/replacement [149].

Remodelling of the podocyte actin cytoskeleton is also a critical step in glomerular injury in multiple animal models and in human disease [96, 218-222]. A common morphological feature of podocyte injury is the replacement of foot processes by squamous areas of cytoplasm, a process known as foot process effacement [223]. Foot process effacement is widely viewed as a pathological derangement that is associated with leakage of
macromolecules, such as albumin, through the glomerular filtration barrier. As a classical adaptive change in podocyte phenotype, foot process effacement is observed in both animal models and human glomerular diseases, including minimal change disease, membranous nephropathy and IgA nephropathy [224].

In 2005, Wharram et al. [225] described a transgenic rat model in which the human diphtheria toxin receptor was specifically expressed in podocytes in order to achieve dose-dependent podocyte depletion. This landmark study showed that while death of less than 40% of podocytes lead to reversible reductions in renal function and transient proteinuria, death of more than 40% of podocytes resulted in segmental sclerosis with sustained proteinuria and reduced renal function. However, more importantly, this study provided evidence that direct podocyte injury was sufficient and had a threshold for the development of progressive glomerular disease. In 2007, Wiggins [226] proposed a podocyte depletion theory as a unifying concept of glomerular diseases that once again places the podocyte as a key cell in glomerular pathology.

As shown in Figure 6, podocyte depletion can be either absolute or relative. While absolute podocyte depletion implies loss, dysfunction or deformation of podocytes (reduction in the number of healthy podocytes), relative podocyte depletion does not involve an absolute reduction in the number of healthy podocytes. Rather, relative podocyte depletion is usually defined as a reduction in the number of podocytes per unit of glomerular volume (i.e. podocyte density). Thus, valid methods for quantifying podocyte number and glomerular volume are required to fully assess podocyte depletion.
1.4.3 Methods to assess podocyte depletion

A range of methods has been employed to estimate podocyte number. Perhaps the most commonly used method involves the counting and reporting of numbers of podocyte nuclear profiles (cross-sections of podocyte nuclei). While this approach may appear valid, the number of podocyte nuclear profiles per glomerular cross-section is not only related to the number of podocyte nuclei present, but also to podocyte nuclear shape and size, as well as section thickness. Moreover, this method does not provide an estimate of the total number of podocytes in a glomerulus. It is therefore of limited value when assessing podocyte depletion [227].

To overcome the problems above, a range of stereological methods were developed in the twentieth century to count cells in tissues. These methods include those of Floderus and
Flex [228], Abercrombie [229], DeHoff and Rhines [230] and Weibel and Gomez [231]. While these methods provide estimates of total podocyte number per glomerulus, they are designated as “model-based” because they require knowledge of the geometry (size, size distribution, shape) of podocyte nuclei. Generally, values for these geometric features are assumed rather than measured, and these assumptions can introduce a certain degree of bias to the values obtained.

With the report of the disector stereological principle [232], these geometric assumptions were no longer necessary. Several groups have applied the disector principle to successfully count podocytes using light [233] and electron microscopy in rat [234] and human glomeruli [235, 236]. In 1986, Gundersen et al. [237] described the optical disector, with which optical sections through tissue sections are compared and particles of interest, such as podocyte nuclei, are counted using the disector principle.

Lemley et al. [227] recently reviewed methods for counting podocytes and described five valid methods: exhaustive enumeration, the Weibel and Gomez method, the disector/Cavalieri combination, the disector/fractionator combination, and the thick-and-thin section method. The disector/fractionator combination was recommended as the preferred method when sufficient tissue was available. The development of the fractionator/disector combination for counting podocytes in glomeruli of known volume is described in Chapter 6. This method involves serial sectioning, immunohistochemistry, confocal microscopy and the application of the disector/fractionator principle to count podocytes, combined with the disector/Cavalieri principle to sample glomeruli and estimate glomerular volume.
1.4.4 Previous studies of human podocyte number

Despite the great interest in podocyte biology, the roles of podocytes in disease initiation and progression, and the podocyte depletion hypothesis, very few studies have reported counts of podocytes in human glomeruli. **Tables 2 and 3** summarize the previous studies of human podocyte number and provide the data obtained. These studies can be classified into two groups depending on the endpoint pathology: T2D and others (which includes T1D, hypertensive nephrosclerosis and IgA glomerulopathy).

**Table 2** summarizes studies that had podocyte number as the main endpoint and provides details on the methods used to estimate podocyte number and glomerular volume, as well as the major findings of each study. In summary, all studies associated podocyte injury/depletion, even at early stages, with progression of disease and markers of glomerular injury (albuminuria). The methods used to quantify podocyte number and glomerular volume differed significantly, and included model-based approaches such as the Weibel and Gomez (for podocyte number and glomerular volume) and mean planar area (for glomerular volume), and design-based approaches such as the Cavalieri method (for glomerular volume) and the disector/fractionator estimator (for podocyte number).

Differences in the estimates of podocyte number and glomerular volume obtained in these studies are almost certainly due, at least in part, to the different methods employed. Before considering the podocyte counts obtained, it is also important to note that a variety of fixatives and embedding media were used in these studies. Shrinkage of kidney tissue varies widely with tissue processing and embedding techniques with 40-50% shrinkage in paraffin and 10-12% shrinkage in resin (such as Epon) [238, 239]. These artefacts can affect the quantification of podocyte number and glomerular volume.
<table>
<thead>
<tr>
<th>Authors</th>
<th>Group</th>
<th>N</th>
<th>Method&lt;sub&gt;PN&lt;/sub&gt;</th>
<th>Method&lt;sub&gt;GV&lt;/sub&gt;</th>
<th>Embedding</th>
<th>Conclusion</th>
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<td>Pagtalunan et al. (1997) [240]</td>
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<td></td>
<td>Podocyte loss contributes to disease progression</td>
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<tr>
<td>Meyer et al. (1999) [241]</td>
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<td>Podocyte depletion contributes to disease progression</td>
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<tr>
<td>Dalla Vestra et al. (2003) [242]</td>
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<td>Podocyte injury at early stages and association with albuminuria</td>
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<td></td>
<td></td>
<td>Podocyte density more functionally relevant</td>
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<tr>
<td>Su et al. (2010) [243]</td>
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<td></td>
<td>Podocyte injury at early stages and association with proteinuria</td>
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<tr>
<td>Other Pathologies</td>
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<td>Steffes et al. (2001) [183]</td>
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<td></td>
<td>Podocyte depletion and functional abnormalities</td>
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<td></td>
<td>Podocyte cross-talk with other cell types</td>
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<tr>
<td>White et al. (2002) [235]</td>
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<td>Early cellular hyperplasia related to glomerular hypertrophy</td>
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<td>Glomerular hypertrophy with podocyte depletion</td>
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<td></td>
<td></td>
<td></td>
<td>Podocyte loss contributes to disease progression</td>
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<tr>
<td>Wang et al. (2009) [245]</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Role of podocyte loss in hypertensive nephrosclerosis</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Authors</th>
<th>Group</th>
<th>Age (Years)</th>
<th>Podocyte number (Cells per glomerulus)</th>
<th>Glomerular Volume (x10^6 µm^3)</th>
<th>Podocyte density (Podocytes per 10^6 µm^3)</th>
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</thead>
<tbody>
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<td><strong>Type 2 Diabetes</strong></td>
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<tr>
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<td>43</td>
<td>512</td>
<td>6.9</td>
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<tr>
<td></td>
<td>MA</td>
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<td>464</td>
<td>6.9</td>
<td>67</td>
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<tr>
<td></td>
<td>DN</td>
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<td>351</td>
<td>8.5</td>
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<tr>
<td></td>
<td>CX</td>
<td>34</td>
<td>575</td>
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<td>235</td>
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<td></td>
<td>DN</td>
<td>59</td>
<td>554</td>
<td>5.4</td>
<td>103</td>
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<tr>
<td></td>
<td>CX</td>
<td>NP</td>
<td>833</td>
<td>3.5</td>
<td>238</td>
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<tr>
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<td>49</td>
<td>579</td>
<td>3.2</td>
<td>181</td>
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<tr>
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<td>DN</td>
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<td>510</td>
<td>3.9</td>
<td>131</td>
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<tr>
<td></td>
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<td>401</td>
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<td>98</td>
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<tr>
<td></td>
<td>CX</td>
<td>NP</td>
<td>825</td>
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<tr>
<td>Steffes et al. (2001) [183]</td>
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<td>16</td>
<td>712</td>
<td>2.3</td>
<td>310</td>
</tr>
<tr>
<td></td>
<td>^DN</td>
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<td>735</td>
<td>3.8</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td>^CX</td>
<td>16</td>
<td>929</td>
<td>2.7</td>
<td>344</td>
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<tr>
<td></td>
<td>^CX</td>
<td>41</td>
<td>878</td>
<td>3.9</td>
<td>225</td>
</tr>
<tr>
<td>White et al. (2002) [235]</td>
<td>MA</td>
<td>38</td>
<td>536</td>
<td>3.6</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>DN</td>
<td>38</td>
<td>502</td>
<td>4.6</td>
<td>109</td>
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<td>CX</td>
<td>38</td>
<td>580</td>
<td>3.2</td>
<td>181</td>
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<td>Lemley et al. (2002) [244]</td>
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<td>85</td>
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<td>CX</td>
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<td>300</td>
<td>2.0</td>
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</table>


**Table 3** provides the estimates of podocyte number and glomerular volume from each of the studies and also provides estimates of mean podocyte density: some of these values were published and the others have been calculated by dividing the published values of mean podocyte number by mean glomerular volume. As seen in **Tables 2 and 3**, each study was designed to compare a particular glomerular disease against respective control groups that varied depending on each study’s respective major endpoint. Among control groups, mean
podocyte number varied from 300 to 929 per glomerulus (a 3.1-fold range), mean glomerular volume ranged from 2.0 to 3.9 x10^6µm^3 (a 1.9-fold range), and podocyte density ranged from 150 to 344 podocytes per x10^6µm^3 (a 2.3-fold range).

The studies of T2D summarized in Tables 2 and 3 showed that podocyte injury, assessed by absolute podocyte number per glomerulus, was present at early stages of diabetic nephropathy and was closely related to disease progression [240-243]. Similar conclusions were drawn from the studies of IgA nephropathy and hypertensive nephrosclerosis [244, 245]. Furthermore, the studies on patients with T1D showed that podocytes may influence the activity and hyperplasia of other cell types (i.e. endothelial and mesangial cells), which is closely associated with glomerular hypertrophy [183, 235].

Glomeruli from patients with T2D contained between 50% and 106% fewer podocytes than control glomeruli. Moreover, podocytes in diabetic glomeruli supported up to 457% more glomerular volume than in controls, which suggests a dynamic interaction between absolute and relative podocyte depletion in diabetic subjects.

Altogether, podocyte depletion appears to play an important role in the development and progression of T2D. More subtle differences were found in glomeruli from subjects with T1D and IgA nephropathy compared to healthy controls – podocyte number was 16-30% lower in these patients compared to controls, and podocyte density was 70-78% lower. These differences in podocyte metrics between patients with T2D and other glomerulopathies may be due in part to age differences (younger patients in T1D and IgA nephropathy studies), different degrees of podocyte injury, time of untreated disease (with T2D being asymptomatic for years before diagnosis) and differences in cell counting methodology. It is worth noting that these studies also highlight the importance of reliable quantification of parameters to define absolute and relative podocyte depletion.
The studies presented in Chapters 6 and 7 aimed to provide numbers of podocytes, endothelial cells, mesangial cells and PECs in individual glomeruli of known volume in adults without renal disease. The studies also provided data for various indices of relative podocyte density, namely podocyte density (number per unit volume of glomerular tissue), and the ratio of podocytes to other glomerular cell types (such as PECs). Studies were also conducted in four children, to provide the first insights into podocyte endowment and podocyte density in children, and to determine whether numbers of podocytes might increase in humans during childhood and adolescence. The presentation in this thesis of a normal range of human podocyte number is the first step towards a comprehensive understanding of human podocyte depletion.

Overall, despite the fact that the podocyte depletion hypothesis has emerged in recent years as a unifying concept in glomerular pathology, very few reliable studies of podocyte number in human kidneys have been conducted. Importantly, the podocyte depletion hypothesis provides an excellent complement to the hyperfiltration theory, unifying a common compensatory response such as glomerular hypertrophy with podocyte depletion, the proposed culprit of progressive glomerular disease in many settings.

### 1.5 General hypothesis

Glomerular hypertrophy is an important response in both renal physiology and pathology. The general hypothesis for the experimental studies described in this thesis is that glomerular hypertrophy in subjects without renal disease is multifactorial, compensatory and associated with relative podocyte depletion. The findings from the experimental chapters in this thesis support this general hypothesis.
References


Chapter 2

Estimating individual glomerular volume in the human kidney: clinical perspectives

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Design of experiment, data analysis, and writing of multiple drafts</td>
<td>80%</td>
</tr>
</tbody>
</table>

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

<table>
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<th>Name</th>
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<th>Extent of contribution (% for student co-authors only)</th>
</tr>
</thead>
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<tr>
<td>Zimanyi, MA</td>
<td>Data collection</td>
<td></td>
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<tr>
<td>Samuel, T*</td>
<td>Data collection</td>
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</tr>
<tr>
<td>Hughson, MD</td>
<td>Tissue collection</td>
<td></td>
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<tr>
<td>Douglas-Denton, RN</td>
<td>Data collection</td>
<td></td>
</tr>
<tr>
<td>Bertram, JF</td>
<td>Experimental design, editing of multiple drafts</td>
<td></td>
</tr>
<tr>
<td>Armitage, JA</td>
<td>Experimental design, editing of multiple drafts</td>
<td></td>
</tr>
</tbody>
</table>

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate’s and co-authors’ contributions to this work*.

<table>
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</table>

*Note: Where the responsible author is not the candidate’s main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.
Estimating individual glomerular volume in the human kidney: clinical perspectives

Victor G. Puelles\textsuperscript{1}, Monika A. Zimanyi\textsuperscript{1}, Terence Samuel\textsuperscript{1}, Michael D. Hughson\textsuperscript{2}, Rebecca N. Douglas-Denton\textsuperscript{1}, John F. Bertram\textsuperscript{1} and James A. Armitage\textsuperscript{1}

\textsuperscript{1}Department of Anatomy and Developmental Biology, Monash University, Melbourne, Australia and \textsuperscript{2}Department of Pathology, University of Mississippi Medical Center, Jackson, MS, USA

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Abstract

Background. Measurement of individual glomerular volumes (IGV) has allowed the identification of drivers of glomerular hypertrophy in subjects without overt renal pathology. This study aims to highlight the relevance of IGV measurements with possible clinical implications and determine how many profiles must be measured in order to achieve stable size distribution estimates.

Methods. We re-analysed 2250 IGV estimates obtained using the discector/Cavilieri method in 41 African and 34 Caucasian Americans. Pooled IGV analysis of mean and variance was conducted. Monte-Carlo (Jackknife) simulations determined the effect of the number of sampled glomeruli on mean IGV. Lin’s concordance coefficient ($\tau_c$), coefficient of variation (CV) and coefficient of error (CE) measured reliability.

Results. IGV mean and variance increased with overweight and hypertensive status. Superficial glomeruli were significantly smaller than juxtaglomerular glomeruli in all subjects ($P < 0.01$), by race ($P < 0.05$) and in obese individuals ($P < 0.01$). Subjects with multiple chronic kidney disease (CKD) comorbidities showed significant increases in IGV mean and variability. Overall, mean IGV was particularly reliable with nine or more sampled glomeruli ($\tau_c > 0.95$, $<5\%$ difference in CV and CE). These observations were not affected by a reduced sample size and did not disrupt the inverse linear correlation between mean IGV and estimated total glomerular number.

Conclusions. Multiple comorbidities for CKD are associated with increased IGV mean and variance within subjects, including overweight, obesity and hypertension. Zonal selection and the number of sampled glomeruli do not represent drawbacks for future longitudinal biopsy-based studies of glomerular size and distribution.

Keywords: glomerular heterogeneity; glomerular size; glomerular volume

Introduction

Chronic kidney disease (CKD) is a major health problem worldwide, with glomerular pathology accounting for $>50\%$ of CKD [1]. Glomerular enlargement is a common feature in several prevalent pathologies including hypertension [2, 3], diabetes mellitus [4, 5] and obesity [6]. Indeed, human mean glomerular volume ($V_{gh}$) is inversely correlated with age [7] and total nephron number ($N_{th}$) [7, 8] and directly correlated with body surface area (BSA) [7, 8] and glomerulosclerosis [9, 10]. Several morphometric methods have been used to estimate $V_{gh}$ [11, 12, 13]. Regardless of the method used, a single estimate of glomerular volume for an entire kidney is obtained. Because the structure and function of human glomeruli varies with their location in the cortex [14, 15], and human kidneys contain on average 900 000 nephrons with a reported 13-fold range (121 000 to 2.7 million nephrons) [16], a single estimate of glomerular volume for a kidney provides a limited insight of glomerular dimensions.

We have previously described a method for the unbiased estimation of individual glomerular volume
Clinical perspectives of IGV [18]. The method involves dissecting sampling of glomeruli and volume estimation using the Cavalieri principle [17, 19]. With this approach, associations between glomerular volume heterogeneity within single kidneys and relevant clinical features can be investigated [11, 20]. Reliable non-invasive methods for in vivo measurements of IGV and its distribution in humans are unavailable, although magnetic resonance imaging methods are available for ex vivo rat kidneys [21]. Thus, human studies currently depend on renal autopsy or biopsy samples for studying the course of glomerular hypertrophy. To date, few studies have used the unbiased dissect科尔-Cavalleri combination to measure IGV in renal biopsies [22, 23], mostly due a lack of (i) understanding as to which cortical zones should be sampled; (ii) the number of glomeruli needed to sample in order to get a reliable estimate and (iii) the financial burden and time-consuming nature of the process often favours other methods [11, 24]. Our previous autopsy-based studies of IGV have sampled 30 glomeruli, 10 from each cortical zone (superficial, middle and juxtamedullary). However, it is not known whether reliable IGV estimates can be obtained when only limited tissue is available, as is the case with renal biopsies. This study is an in situ analysis of our IGV measurements of 75 American subjects, the largest database available. We provide data that supports the relevance of IGV estimates through an analysis of accepted commodities for CKD. To provide guidelines for future biopsy studies, we explored IGV mean and variance in different cortical zones and determined how many glomeruli must be measured to generate reliable mean IGV estimates.

Materials and methods

Tissue

Tissue was obtained at coronal autopsies performed at the University of Mississippi Medical Center (Jackson, MS) between 1998 and 2005. Permission for autopsy was obtained from next-of-kin and ethical approval was provided by the respective boards of the University of Mississippi and Mississippi University. Right kidneys were perfusion-fixed with 10% buffered formalin, biopsied and immersed in 10% formalin. After 10 days, both halves were cut into slices 4 mm thick and every fourth slice of both halves sampled for stereology. Selected slices were sent to Monash University for analysis.

Subjects

We reviewed data from 75 American males (41 African Americans and 34 Caucasians) who were included in previous publications [17, 25, 26]. Eligible subjects were included in our Monash University Renal Autopsy Database. Clinical data were obtained from the Forensic report, emergency department and hospital records. Variables such as body mass index (BMI) and BSA were calculated based on weight and height. Subjects were also categorized as hypertensive or normotensive based on a number of different criteria including confirmed blood pressure from medical records and histopathology [17, 27].

Other sub-groups were also defined: subjects were classified as obese with a BMI > 30.0 kg/m² and lean with a BMI ≤ 24.9 kg/m². We further defined BMI sub-groups based on accepted commodities for CKD: low-risk (age ≥ 30 years, BMI ≤ 29.0 kg/m² and no evidence of hypertension) and high-risk (age ≥ 60 years, BMI ≥ 30.0 kg/m² and the evidence of hypertension).

Estimation of total glomerular number (N_{G},mm)

Total nephron (glomerular) number was estimated using the physical dissector/summation combination as previously described [8]. This is a design-based stereological method that provides unbiased estimates. Macroscopically, kidney tissue was systematically sampled, embedded in glycol methacrylate and exhaustively sectioned at 20 µm. Section pairs were used to count glomeruli at a unique point in a known fraction of the kidney. Total glomerular number was calculated using basic algebra.

Estimation of IGV

The technique used to estimate IGV has previously been described in detail [18]. Briefly, a tissue slice (∼10 x 10 x 1 mm) containing full thickness cortex and medulla was obtained from the mid-nephron region. After glycolmethacrylate embedding [28], blocks were exhaustively sectioned at 10 µm and stained with periodic acid-Schiff. Slides were projected onto a white surface using an Olympus BH-2 microscope at a magnification of x320. Three to four sections were analysed: outer (superficial) located within four glomerular diameters of the capsule; inner (juxtamedullary) located within four glomerular diameters of the arcuate vessels and mid-cortical zone as intermediate between the outer and inner zones. Thirty glomeruli (10 per zone) in each kidney were sampled using dissectors [29] and their volumes estimated using the Cavalieri method [30]. Each glomerulus used for volume estimation was exhaustively sectioned at a 10-µm. The glomerular tuft area of every second section was measured by point counting using an orthogonal test grid (area per point = 1 cm² at a final magnification of x320). IGV was represented by the glomerular tuft volume and was estimated using:

$$\text{IGV} = \sum_{i=1}^{n}\text{V}_{\text{G,mm}} \times \text{SSF} \times \pi$$

where $\text{V}_{\text{G,mm}}$ is the total number of grid points overlying the glomerular tuft, $\pi$ is section thickness (10 µm) and SSF is the reciprocal of the section sampling fraction, in this case 2 because every second section was analysed.

Statistical analysis

Statistical analyses were performed using Stata (version 8), and statistical significance defined as a $P < 0.05$.

The general features of variables were described in mean, SD, minimum and maximum values. To assess distribution of IGV values within subjects, the Shapiro–Wilk W test was used and skewness defined when statistical significance was observed. Student's t-test or analysis of variance tests were performed whenever applicable. For skewed variables, Mann–Whitney and Kruskal–Wallis tests were applied. For zonal analysis, the pooled mean IGV, SD, range (maximum value minus minimum value) and variance were estimated in order to assess differences between cortical zones.

A gold standard IGV mean value was defined from the 30 sampled glomeruli per subject (10 glomeruli per zone). Then, Monte-Carlo simulation (Jackknife) was performed in order to simulate the effect of reducing the number of sampled glomeruli (from 29 to 3 sampled glomeruli per subject). This approach provided 27 raw mean IGV values, which were compared with the gold standard mean IGV. This process was repeated three times in order to compute the variability generated by random selection. Our database contains IGV values for 2250 glomeruli from 75 well-matched subjects and therefore provides a greater power than which might be achievable in many settings. In order to simulate the effect of measuring fewer glomeruli in a smaller cohort size, a random sample of 10 subjects per race was selected and the effect of the Jackknife evaluated.

The coefficient of variation (CV, 100 × SD/mean IGV) and the coefficient of error (CE, 100 × SE/mean IGV) were calculated for each mean IGV generated by Jackknife simulation. Lin’s concordance coefficient ($R_{c}$) [11] was used to assess precision, accuracy and the 95% limits of agreement of mean IGV values from the Jackknife simulation; again, to determine whether reliable estimates of IGV size distribution could still be achieved whilst measuring fewer glomeruli. Finally, a regression analysis was conducted between $R_{c}$ and different mean IGV values. $R_{c}$ was selected because of its previously described inverse linear relationship with mean glomerular volume [8]. We tested whether this relationship was preserved when fewer glomeruli were measured.
Table 2. Zonal analysis comparing the mean and variance of IGVs for male African and Caucasian Americans

<table>
<thead>
<tr>
<th>Group</th>
<th>Zone</th>
<th>Number of glomeruli measured</th>
<th>Mean IGV ($\times 10^5 \mu m^2$)</th>
<th>IGV variance ($\times 10^5 \mu m^2$)</th>
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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>CA</td>
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</tr>
<tr>
<td></td>
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<td>410</td>
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<td>4.21$^a$</td>
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$^a$ Group definitions: AA (African Americans), CA (Caucasian Americans), NT (normotensive), HTN (hypertensive), low (BMI $\geq$33.8 kg/m$^2$), obese (BMI $\geq$30.0 kg/m$^2$), low (age $\leq$ 60 years + BMI $\leq$28.9 kg/m$^2$ + normotensive) and high (age $\geq$ 60 years + BMI $\geq$30.0 kg/m$^2$ + hypertensive). Zones: S (superficial), M (middle) and J (juxtamedullary). Numbers in italics of bold type are statistically significant.

$^b$ P < 0.001 between comparable groups and within the same zone.

$^c$ P < 0.05 between comparable groups and within the same zone.

zones (see Table 2). Superficial glomeruli were significantly smaller than juxtamedullary glomeruli (all subjects, $P < 0.01$) and by racial group ($P < 0.05$). However, in lean, normotensive and low-risk subjects IGV mean and variance did not differ between the three zones. Interestingly, there was no association between zonal location and IGV mean or variance in hypertensive individuals nor did subjects at high-risk of kidney disease demonstrate location-specific changes in IGV (P > 0.05). In contrast, middle and juxtamedullary glomeruli from obese individuals, especially among obese Caucasians, showed larger and greater IGV variability (Table 2).

Notwithstanding the minimal effects of cortical zone, hypertensive individuals demonstrated IGVs that were significantly larger than normotensives (P < 0.001) and more heterogeneous (P < 0.001) in all three zones. A similar pattern was observed when comparing risk of kidney disease groups, where glomeruli from low-risk subjects were smaller and more homogeneous across all cortical zones than their high-risk counterparts (Table 2).

Optimizing technical efficiency

Following the Jackknife procedure to simulate the effects of sampling fewer than 30 glomeruli per subject, 27 new mean IGV values were generated for both racial groups (Table 3). Surprisingly, average IGV was barely altered by reducing the number of glomeruli measured, with little variation within and between groups. Mean IGV in African Americans (n = 41) was as stable as in Caucasian Americans (n = 34), even when the African Americans presented a larger mean IGV and more heterogeneous distribution. In African Americans, CV ranged from 29.02% (30 glomeruli measured per subject) to 29.33% (3 glomeruli measured) and CE from 4.53% (30 glomeruli measured) to 4.58% (3 glomeruli measured), whereas in Caucasian Americans, CV ranged from 32.1% (30 glomeruli measured) to 32.20% (3 glomeruli measured) and CE from 5.52% (30 glomeruli measured) to 5.52% (3 glomeruli measured). When 10 subjects were randomly sampled in each racial group, mean IGV for each subject showed little variation when more than nine glomeruli were measured per subject (Figure 2). Moreover, $R_2$ and the 95% limits of agreement were especially strong when more than nine glomeruli were measured per subject for each racial group (Figure 3). Figure 4 shows the linear relationship between mean IGV and $N_{glomer}$ The general trend was preserved with all the different glomerular sample sizes (from 29 to 3 glomeruli measured per subject, data not shown) in both racial groups. The linear regression was statistically significant when eight or more glomeruli were measured from Caucasian Americans and nine or more glomeruli were measured from African Americans. Together, the results of the Lin's concordance, Bland and Altman and linear regression analyses suggest that a stable estimate of mean IGV and IGV distribution are achieved where more than nine glomeruli are sampled per subject.

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Table 3. Mean IGV values generated by the Monte-Carlo Jackknife simulation

<table>
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<tr>
<th>Number of sampled glomeruli per subject</th>
<th>African: Amerindian (n = 48) Mean (SD) X 10^6 µm³</th>
<th>CV (%)</th>
<th>CE (%)</th>
<th>Caucasian: Amerindian (n = 34) Mean (SD) X 10^6 µm³</th>
<th>CV (%)</th>
<th>CE (%)</th>
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<tr>
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<td>(µm³)</td>
<td>(µm³)</td>
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<td>(µm³)</td>
<td>(µm³)</td>
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<tr>
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<td>29.02 ± 4.53</td>
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6Number of sampled glomeruli per subject, number of glomeruli randomly sampled by the Monte-Carlo simulation; min, maximum volume (X 10^6 µm³); CV, coefficient of variation (100 X SD/mean); CE, coefficient of error (100 X SE/mean).

Discussion

This study reinforces the clinical relevance of IGV measurement, demonstrating that IGV mean and variance increase with established CKD comorbidities including age, obesity and hypertension. It also illustrates how reliable estimates of mean IGV can be obtained with at least nine sampled glomeruli.

This is the largest cohort study to show associations between obesity and hypertension and IGV. Hypertension and obesity are major contributors to the CKD epidemic, especially in populations with increased risk of kidney disease including Australian Aborigines [32], Pima Indians [33] and African Americans [34]. Of relevance to the present study, glomerular hyper trophy has been described in all these populations [32, 33, 35, 36]. Glomerular size and size variability are the two important markers of glomerular stress [16] and age [18, 37], BMI, hypertension [38] and NACs [17, 26, 37] may act as glomerular stressors, increasing mean IGV and heterogeneity.

Glomerular morphology and function vary according to their location within the renal cortex [39]. Skov et al. [15] showed that in healthy primate, juxta-medullary glomeruli were up to four times larger than superficial glomeruli and our first autopsy study of IGV showed that superficial glomeruli were larger than juxta-medullary glomeruli in advanced age (>50 years) and high BSA (>2.11 m²) [18]. Our current analysis considered 2250 glomeruli from subjects with minimal comorbidities (lean, normo-tensive and low risk of kidney disease) and observed no zonal differences. Low-risk individuals presented smaller more homogeneous glomeruli in all three cortical zones compared with high-risk subjects. Moreover, middle and juxta-medullary glomeruli from high-risk subjects tended to be larger than superficial glomeruli (8-13%), a tendency that was not observed in the low-risk group. The data from the low-risk kidneys are in agreement with two human autopsy studies; neither Zimanyi et al. [26] nor McNamara et al. [37] found zonal differences in IGV, despite analysing 48 adult males (24 American and 24 Senegalese) and a total of 1440 glomeruli. Furthermore, Newbold et al. [40] found no differences in the ratio of juxta-medullary and superficial glomeruli in nonscocial human necropsies [7].

Tracey [41] suggested that glomerular size heterogeneity could be closely related to vascular changes, especially in hypertensive nephrosclerosis. Hoy et al. [42] showed that among subjects with hypertension, wall thickening of interlobular arteries was present at a younger age than the increase in glomerulosclerosis, suggesting that vascular changes preceded parenchymal loss. Moreover, the scenario of hyperfiltration (such as obesity or any other type of parenchymal loss), renal blood flow would need to
be re-distributed to preserve function and glomeruli closer to principal vessels (juxtaglomerular) would possibly be favoured by their proximity to the blood supply. As described by Brenner [43], this hyperfiltration process would lead to progressive glomerular hypertrophy and finally glomerulosclerosis in the remaining glomeruli. Hoy et al. [18] proposed that small glomeruli may be more likely to sclerose and Samuel et al. [18] found that global glomerulosclerosis increased with age and was most severe in the superficial cortex. This observation is supported by our findings in high-risk subjects with smaller glomeruli in the superficial area. Whether this difference between superficial and juxtaglomerular glomeruli reflects mostly vascular changes, or structural differences between glomeruli such as differences in podocyte number, is still to be defined.

We and others have estimated mean glomerular volume in renal biopsies [44, 45], but few studies have assessed variability in glomerular volumes within single biopsies with unbiased stereology [22, 47]. The method of Weibel and Gomez (1962) has been most commonly used to estimate glomerular volume in biopsies but requires knowledge of glomerular size distribution and shape [22, 44]. While the effect of size distribution has been previously addressed [48], the effect of assuming a uniform glomerular shape remains unknown and may be a source of significant bias.

We propose that measurements of IGV in renal biopsies would provide valuable insights in the diagnosis, prognosis and management of CKD [49]. However, several caveats must be mentioned. While biopsies are usually taken from the lower pole of the kidney, our

Fig. 2. The effect of reduced glomerular sampling (through Jackknife simulations) on mean IGV values. Each dot represents one mean IGV value, dotsotted by a non-contiguous line correspond to one subject. (A) (African Americans, n = 10 subjects) and (B) Caucasian Americans, n = 10 subjects) represent the variation between mean IGV generated with 30 sampled glomeruli (open circles at the beginning of each horizontal line) and Jackknife generated values (black dots, from 29 to 3 sampled glomeruli per subject).

Fig. 3. Lin’s concordance coefficient (Rc) and its respective limits of agreement (LA, 95% CI) for different mean IGV values generated by the Jackknife at African Americans (Column A, n = 31) and Caucasian Americans (Column B, n = 34). The horizontal lines (——) illustrate 2 SD or ±1 × 10^14 μm^3 and the vertical Lines (——) show the perfect agreement for Rc.
estimates of IGV have been made in tissue from the mid-literal region of the kidney. Lodrup et al. [50] report no differences in mean glomerular volume between mid-literal region and renal poles in pigs but this has not been addressed in humans. Furthermore, biopsy samples have a preponderance of superficial glomeruli because of the risks of sampling in the juxtamedullary zone. Superficial glomeruli demonstrate changes in volume and volume distribution with disease but are stable under normal conditions, supporting their use for monitoring kidney health longitudinally.

The stereological dogma of ‘do more, less well’ may also be applicable to measurements of IGV [13, 51]. Although there are obvious advantages of designing studies in which as many glomeruli as possible are measured, our observation that a relatively modest number of glomeruli must be measured to return a reliable estimate of IGV mean and distribution suggests that longitudinal biopsy-based studies may be feasible. An average of 9–12 glomeruli [52] can be obtained from a percutaneous needle biopsy and the present study suggests that reliable estimates of mean IGV could be obtained with at least nine ‘complete’ or dissector-sampled glomeruli per subject. Group analysis (mean IGV by racial group) and individual analysis (comparison of mean IGV values in 10 randomly sampled subjects per racial group) returned similar results, suggesting that the stability of IGV estimates is not a function of the large number of subjects available for this study.

Another relevant issue is the time-consuming nature of IGV estimation. MacLeod et al. [22] reported that five glomeruli per biopsy at a sectioning interval of 20 µm
would provide satisfactory estimates of mean glomerular volume by the Cavalieri principle in normotensive type 1 diabetic patients. The present findings in subjects without overt renal pathology indicate that even three glomeruli per subject would barely alter the mean IGV, CV and CE compared with that observed from 30 glomeruli. However, valuable information about the spread or distribution of IGVs, the ability to perform correlations and statistical power would be lost with reductions below nine glomeruli per subject. Moreover, if the research question extends to an assessment of glomerular size distribution and variance within and between subjects, then the measurement of as many complete glomeruli as possible should be encouraged. Furthermore, new technologies such as the use of digital images and stereology-based software offer improvements in efficiency.

The present study has a number of limitations. Firstly, the IGV values utilized came from subjects carefully selected for various criteria in previous studies [18, 26]. Notwithstanding this, the matching criteria set previously were mainly based on age. Secondly, subjects in the three previous studies were selected for different end points, based on the extremes of the distributions for each variable of interest (age, Nephron hypertension and BMI). Though this may appear to introduce a selection bias, we consider that our results based on subjects on the lower and upper end of their distributions would restrain our optimization process up to a sensible point. All glomeruli measured were contained in a mid-hilar slice of tissue. As such, any differences in the size or size distribution of glomeruli in the renal poles were not taken into account. Another important caveat would be the differences in the fixation process. Biopsy tissue is immersion-fixed, whereas we studied perfusion-fixed tissue. Mean glomerular capillary volume is lower in immersion versus perfusion fixed tissue [53]. Interestingly, Maclod et al. [22] have used the Cavalieri method to estimate glomerular volume in immersed-fixed human biopsy tissue, from diabetic individuals and showed a similar range of glomerular volumes to our present data. This outcome further strengthens the argument for estimating variability in glomerular volume in the study of renal disease.

In summary, this large cohort study of 2250 glomeruli from 75 individuals provides further evidence that the size distribution of glomeruli throughout the renal cortex is a relevant marker of risk factors for KD in including obesity, hypertension and low nephron number. Neither glomerular location within the renal cortex nor the number of sampled glomeruli per subject represent significant drawbacks for the application of IGV to tissue samples of limited size, including renal biopsies.

Funding

This research was funded by grants from the National Institutes of Health (NH 1 R01 DK065970-01), the NIH Center of Excellence in Minority Health (SP01M000534-02), the National Health and Medical Research Council of Australia (NHMRC) and the American Heart Association (Southeastern Affiliate). J.A.A. is a Monash Fellow and PhD scholarship funding for V.G.P. was provided by Monash Research Graduate School (MRGS) and Faculty of Medicine International Postgraduate Scholarship.

Conflict of interest statement. None declared.

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ou 2008; 452: 393–403

Received for publication: 8/6/11; Accepted in revised form: 12/8/11
Chapter 3

Hypertension, glomerular hypertrophy and nephrosclerosis: the effect of race

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

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<thead>
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<th>Extent of contribution (%)</th>
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<tr>
<td>Data analysis and editing of multiple drafts – 20%</td>
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The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate’s and co-authors’ contributions to this work.

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<th>Main Supervisor’s Signature</th>
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Background. African Americans have more severe hypertensive nephrosclerosis than white Americans, possibly at similar levels of blood pressure. Glomerular volume is increased in African Americans relative to whites, but it is uncertain how this relates to nephrosclerosis and whether it contributes to or compensates for glomerulosclerosis.

Methods. Stereological dissector/fractionator estimates of glomerular number ($\text{N}_{\text{glomer}}$) and average glomerular volume ($\text{V}_{\text{glomer}}$) were obtained on autopsy kidneys of 171 African Americans and 151 whites. Eighty-eight African Americans and 49 whites were identified as hypertensive. Nephrosclerosis was measured morphometrically as the percentage of glomerulosclerosis, proportion of cortical fibrosis and interlobular artery intimal thickness, and analyzed with $\text{V}_{\text{glomer}}$ by age, race, gender, body mass index (BMI) and blood pressure.

Results. African Americans were more frequently hypertensive (58.5%) than whites (35.8%) and when hypertensive had higher levels of blood pressure ($P = 0.02$). $\text{N}_{\text{glomer}}$ was significantly lower in hypertensive compared with normotensive subjects among white women ($P = 0.02$) but not white males ($P = 0.34$) or African American females ($P = 0.10$) or males ($P = 0.41$). For each race and gender, glomerulosclerosis, cortical fibrosis and arterial intimal thickening were statistically correlated with age ($P < 0.001$) and hypertension ($P < 0.001$) and increased $\text{V}_{\text{glomer}}$ with hypertension ($P < 0.001$) and BMI ($P < 0.001$). In multivariate analysis, African American race was associated with increased $\text{V}_{\text{glomer}}$ ($P = 0.01$) and arterial intimal thickening ($P < 0.01$), while interactions between race and blood pressure indicated that the severity of nephrosclerosis including increased $\text{V}_{\text{glomer}}$ was linked most directly to hypertension without significant contributions from race. The hypertension-associated enlargement of $\text{V}_{\text{glomer}}$ was present with mild degrees of glomerulosclerosis and changed little as the severity of glomerulosclerosis increased.

Conclusions. Glomerular hypertrophy was identified as an integral feature of hypertensive nephropathy and appeared to precede rather than compensate for glomerulosclerosis. An effect of race on $\text{V}_{\text{glomer}}$ and arterial intimal thickening seemed to be related to the more frequent and more severe hypertension among African Americans.

INTRODUCTION

Hypertension is a disease of aging. It is identified in fewer than 10% of persons under 35 years of age but affects >60% of the US population after age 65 [1, 2]. It is more prevalent and tends to be more severe and to begin at an earlier age in African Americans than whites, and African Americans have a greater risk of end-stage renal disease (ESRD) attributable to hypertension [2, 3, 4].
Hypertension results in a complex of progressive kidney changes recognized as arteriosclerosis, cortical fibrosis, tubular atrophy and loss, and glomerulosclerosis that are pathologically referred to as arteriolar or hypertensive nephrosclerosis [3, 5, 6, 7]. Glomeruli become globally sclerotic in two distinct patterns. One is termed obsolescence and the other solidification [8, 9, 10, 11]. The predominant form of glomerulosclerosis in hypertensive nephropathy is obsolescence that is recognized by collapse of the glomerular tuft and intracapillary fibrosis [7, 12]. Obsolescence is considered to be the result of reduced glomerular perfusion because of arteriosclerotic narrowing of preglomerular arteries and arterioles. Glomerular solidification expands the glomerular tuft by an increased accumulation of mesangial matrix and is proposed to be a complication of glomerular hyperfiltration secondary to a loss of afferent arteriolar autoregulation [8, 9, 10, 11, 13, 14].

A linear concept of the progression of hypertensive nephropathy suggests that the severity of nephrosclerosis, and particularly the extent of glomerulosclerosis, corresponds to a reduction in glomerular filtration rate (GFR). Contrast-enhanced computed tomography imaging of the kidneys of transplant donors have shown an age-related loss of cortical volume that correlates with a reduction in GFR [7, 12, 15]. Despite this tendency, individuals at increased risk of chronic kidney disease by virtue of obesity, lipid abnormalities, hypertension and microalbuminuria demonstrate increases in cortical volume and GFR with mild and moderate nephrosclerosis [7, 12, 15]. In these situations, single nephron filtration rates are increased, and the increase is thought to be the result of a compensatory enlargement in the volume of functioning glomeruli [7, 12, 15].

On the basis of clinical observations and experimental models of nephron deficiency, glomerular hypertrophy is suggested to precede glomerulosclerosis with the glomerulosclerotic being attributed to glomerular hypertension [8, 16, 17]. This concept may explain glomerular solidification but is difficult to reconcile with glomerular obsolescence being the principal form of glomerulosclerosis found in hypertensive nephropathy, at least in its early and intermediate stages [18, 19, 20]. However, it is not at all clear whether glomerular obsolescence is a direct result of reduced glomerular perfusion, or whether arteriosclerosis, glomerular obsolescence and glomerular solidification share a common developmental mechanism [7, 8, 10, 12, 15]. In the later stages of hypertensive nephropathy, glomerular solidification certainly becomes prominent, if not the predominant form of glomerulosclerosis, as renal function decompensates [8, 16, 21].

Renal biopsy and autopsy studies have found that African Americans have larger glomeruli than whites [22–25]. Furthermore, the African American Study of Kidney Disease (AASK) and Hypertension biopsy series of hypertensive nephrosclerosis showed that African Americans had more severe arteriosclerosis and glomerulosclerosis than whites, that glomerular solidification was more frequent and that the severity of the changes could not be attributed to racial differences in blood pressure [9, 10]. These observations suggested that the glomerular hypertrophy of African Americans reflects their greater risk of hypertension-associated kidney disease.

Our research over the last decade has analyzed glomerular number ($N_{glomer}$) and glomerular volume ($V_{glomer}$) in a large sample of kidneys by the fractionator/disector method to test the hypothesis that low $N_{glomer}$ may contribute to higher rates of hypertension among African Americans [18, 19, 20, 26]. The studies have not identified any significant difference in $N_{glomer}$ between hypertensive and non-hypertensive African Americans, and no significant racial difference has been found despite African Americans being much more frequently hypertensive [18, 19, 20, 26]. Throughout the several analyses, an inverse relationship with $N_{glomer}$ was found to be the strongest determinant of $V_{glomer}$. This inverse relationship is seen soon after adult glomerular size is attained and appears to be an inherent feature of kidney growth in which for any level of $N_{glomer}$, total glomerular volume tends toward a middle range [18, 19, 24, 26]. Nevertheless, among adults, African Americans were found to have larger glomeruli than whites despite the similar numbers of glomeruli [18, 19, 24, 26].

The aims of this current study are to evaluate the microanatomical changes of hypertensive nephrosclerosis and their relationships to age, race, gender, body mass index (BMI) and blood pressure. The associations emphasized variations in glomerular volume in an attempt to delineate relationships between $V_{glomer}$ race and glomerulosclerosis.

**MATERIALS AND METHODS**

**Specimen and subject data collection**

This research used human autopsy tissue. The study was approved by the Institutional Review Board of the University of Mississippi Medical Center and the Human Research Ethics Committee of Monash University. Permission for autopsy was obtained from first-of-kin.

Right kidneys were collected at autopsy and perfusion fixed with 10% formaldehyde at the University of Mississippi Medical Center (Jackson, MS, USA) between the years 1998 through 2005. The research was a study of glomerular number and volume in persons without known renal disease who, because of their residence in Mississippi, may have been at high risk for primary hypertension and hypertensive vascular disease. The inclusion criteria required that kidneys be: (i) equal or approximately equal in size, (ii) grossly normal or show mild to moderate arteriolonephrosclerosis with a uniformly granular subcapsular cortex. Kidneys from patients with diabetes were analyzed if they showed mild to moderate arteriolonephrosclerosis but were excluded if there was microscopically a diabetic nephropathy. On this basis, kidneys of 11 diabetics were used, 9 African Americans and 2 whites.

The exclusion criteria consisted of: (i) kidneys involved by coarse pits or angular or depressed cortical scars, in order to exclude examples of ischemic nephropathy and pyelonephritis and not to have glomerular number reduced by severe renal scarring and advanced glomerulosclerosis. (ii) Kidneys of patients who had premorbid clinically diagnosed kidney disease. This was never a problem, as patients in this category were autopsied for complications of dialysis and in one instance death due to pulmonary emboli complicating a
membranous nephropathy. (iii) Cases of congenital heart disease including bicuspid aortic valves, hereditary hypertrophic cardiomyopathy, dilated cardiomyopathy and congenital heart disease because normal cardiac function, and blood pressure regulation may not have been present and heart weight would be increased independent of blood pressure.

Included subjects were 1 month to 89 years old. Twenty-two were under 1 year of age and 13 were older than 65. The study cohort consisted of 86 African American males, 94 African American females, 87 white males and 52 white females. The causes of death were coronary artery disease (CAD) and/or hypertensive cardiac disease, 26%; cerebrovascular disease (CVD), 7%; pulmonary embolus, 7%; neoplastic, hematologic or infectious disease, 13%; pulmonary disease, 10%; accident, 12%; homicide, 5%; suicide, 6%; neurological disease not CVD, 3%; unknown, 4%; sudden infant death syndrome, 3%; cardiac not CAD or hypertensive disease, 4%; and complications of prematurity, 1%.

Clinical information was obtained from University of Mississippi Medical Center records and from the investigative reports of the county Medical Examiner. Blood pressures without medication were obtained from 115 African Americans and 67 whites. Blood pressure from terminal hospital admissions were not used unless patients were diagnosed as hypertensive and blood pressures were elevated. Mean arterial blood pressure (MAP) was calculated from an average of at least three blood pressure determinations.

The patients were categorized on the basis of a history of hypertension, consistently elevated blood pressure (≥140/90 mmHg), the presence of cardiomegaly and the severity of intrarenal arteriolar sclerosis as previously described [15, 16]. Patients were also categorized on the basis of diabetes, death due to CAD or CVD and treatment for hypertension. Patients were recorded as treatment for hypertension if there was any statement of such treatment in the medical records or medical examiners’ reports.

**Measurement of total glomerular number (N_{gln}) and mean glomerular volume (V_{gln})**

Kidneys were perfused with 10% buffered formalin and then bisected and immersed in 10% formalin for 10 days. Afterward, both halves of the kidney were cut into slices 4 mm thick and every fourth slice of both halves was sampled for stereology beginning with the first slice selected as a random number from 1 to 4. The selected slices were sent to Monash University where they were processed for embedding in glycol methacrylate for the stereological estimation of total glomerular number (N_{gln}) and mean glomerular tuft volume (V_{gln}, μm³ × 10⁶) using the physical dissector/fractionator method [27].

**Measurement of glomerulosclerosis, cortical fibrosis and arteriolar sclerosis**

Representative kidney blocks from the upper pole and the mid-portion of the kidney not sampled for stereology were paraffin embedded. Sections were cut at 4 μm and stained with periodic acid-Schiff (PAS)-hematoxylin and picric acid red stains for fibrillar collagen.

Measurement of the percentage of glomerulosclerosis and arterial intimal thickening used PAS-hematoxylin stained sections. The percentage of sclerotic glomeruli was estimated by counting sclerosed and non-sclerosed glomeruli in non-overlapping x100 microscopic fields moving from the subcapsular surface to inner cortex with at least 400 glomeruli being counted per subject. The severity of arteriosclerosis was measured as a ratio of the thickness of the intima to the outer wall diameter at a magnification of x100 in interlobular arteries 90–250 μm in diameter using the linear measurement function of Image-Pro Plus morphometric software (Media Cybernetics Inc., Bethesda, MD, USA). Cortical fibrosis was measured in x200 images as the proportion of cortical staining red with the picrosirius stain using the automated Image-Pro Plus area counting function.

**Statistical methods**

Data were collected into Microsoft Excel™ and analyzed with StaTs™ (College Station, TX, USA) software. Multinomial least squares regression was used to evaluate the independent variables of age, race, gender, BMI, CAD/CVD death, diabetes and treatment of hypertension on the dependent structural variables of V_{gln} glomerulosclerosis, cortical fibrosis and arterial intimal thickening. Multinomial regression models were used to evaluate the interactions of V_{gln}, glomerulosclerosis, cortical fibrosis and arterial intimal thickening with race and MAP. To evaluate possible effects of influential outliers, least squares regressions were checked with robust regression models using Huber maximum likelihood and scaled estimators of high-leverage outliers. Differences between groups were analyzed using an F-test if data passed normality and equal variance tests and by a Mann–Whitney rank sum test if they did not. Spearman’s rank-order correlation evaluated pairwise relationships between variables. Discrete variables were compared by χ²-tests. For all statistical procedures, a P-value of <.05 was considered significant.

**RESULTS**

**Age relationships to hypertension and V_{gln}**

Medical Examiner investigations of sudden unexpected deaths accounted for 57% of the autopsies with 51% of these deaths being the result of CAD or CVD. The youngest hypertensive subject at death was 25 years of age. MAP reached hypertensive levels (≥ 130 mmHg) somewhat earlier and the level of blood pressure was higher (P = 0.02) for African Americans than whites. V_{gln} increased during childhood and adolescent growth. Adult size was reached at 18–20 years of age (Figure 1), and from early adulthood to old age the V_{gln} of hypertensive subjects was larger than those without hypertension (P < 0.001).

N_{gln} ranged from 210332 to 2702079 with an average of 918210 ± 326077. For all subjects, females had 10% fewer glomeruli than males with the gender difference being only significant for whites (African American males 941903 ± 351504, n = 86, African American females 877169 ± 328866, n = 94, P = 0.20; white males 986338 ± 314387, n = 87; white females
FIGURE 1: The relationship between $V_{GLO}$ and age by quadratic prediction plots comparing hypertensive and non-hypertensive subjects. $V_{GLO}$ increases with growth during adolescence and early adulthood and then plateaus at age. For hypertensive subjects, $V_{GLO}$ was larger than for non-hypertensive subjects from early adulthood through old age ($P<0.001$).

839.253 ± 294.790, n = 52, P = 0.01). Fewer glomeruli were found in kidneys with more glomerulosclerosis ($r = -0.190$, $P = 0.01$). This modest reduction in glomerular number was the result of the trend for fewer $N_{GLO}$ to be found in patients with increased glomerulosclerosis.

Different patterns of glomerulosclerosis and their relationship to ageing and blood pressure

From infancy to ~3 years of age, up to 8% ‘congenital’ glomerulosclerosis was found [23]. At the end of adolescence, glomerular obsolescence began to be observed. In the early stages, obsolescence was recognized by thickening and wrinkling of the glomerular capillary basement membrane and collapse of the tuft toward the hilar pole. In the late stages, the collapsed tuft became a bright PAS-positive hyaline knot surrounded by intracapillary fibrosis that filled Bowman’s space within a largely intact Bowman’s capsule basement membrane [10, 11]. Occasional perihilar segmental glomerulosclerosis with hyalinosis was found in juxtedudillary glomeruli. These glomeruli were infrequently identified mainly in obese subjects who died of CAD/CVD but in numbers that were too low to enumerate meaningfully in the 400 or more glomeruli counted per subject.

Glomerular solidification was defined by the following features [10, 11]: (i) in the early stages by focal segmental glomerulosclerosis (FSGS) that consisted of segmental effacement of glomerular capillaries by increased mesangial matrix and by adhesions to Bowman’s capsule. (ii) The later stages consisted of consolidated PAS-positive basement membranes and mesangial matrix surrounded by a fragmented Bowman’s capsule basement membrane. This combination of FSGS and solidification was found in three subjects. All three had severe hypertension and severe intimal fibrosis of arterial and interlobular arteries. One subject was a 56-year-old African American male with a MAP of 154 mmHg, 1,028.482 glomeruli and 1.25% glomerulosclerosis. One was a 57-year-old African American male with a MAP of 106 mmHg, 875.516 glomeruli and 1.18% glomerulosclerosis. The third was a 56-year-old white male with a MAP of 152 mmHg, 1.142.909 glomeruli and 27.8% glomerulosclerosis. In each case, glomerulosclerosis consisted of >50% solidified and 50% obsolescent glomeruli.

Early in adulthood, the number of obsolete glomeruli, hereafter referred to as glomerulosclerosis, rarely exceeded 2–3%. At age 30–40, glomerulosclerosis began to increase. The rate of increase depended upon whether or not the subjects were hypertensive and, if hypertensive, on the magnitude of the elevation of blood pressure. For non-hypertensive subjects, glomerulosclerosis was related to age ($P<0.001$) but without a significant contribution from MAP ($P=0.45$). For hypertensive subjects, the percentage of glomerulosclerosis was related to age ($P<0.001$) and MAP ($P<0.001$).

Clinical and kidney structural features of hypertensive and non-hypertensive subjects 18–70 years old

The average age at death for all hypertensive subjects was 48.7 ± 12.2 years with a range of 25–89 years. Fourteen of the 135 hypertensive subjects (10.4%) died with or were identified as having hypertension at or before age 30 (Table 1). The youngest of these 14 patients were 17, 19, and 20 years old. The oldest non-hypertensive person was 69 years old. In order to evaluate $N_{GLO}$ and $V_{GLO}$ in subjects of similar age, comparisons were made between hypertensive and non-hypertensive subjects between 18 and 70 years old. The clinical and pathological features of these subjects are summarized in Table 2.

There were proportionately more hypertensive African Americans than whites ($P<0.001$), and hypertensive African Americans had a greater MAP than hypertensive whites, $P=0.02$. CAD/CVD accounted for 54.7% of deaths among African Americans and 47.8% of white deaths. Nine diabetics African Americans were hypertensive and five died at a median of 52 years of age from CAD/CVD. One of two white diabetics was hypertensive and died at age 37 from CAD/CVD. Proportionately similar numbers of subjects of both races were treated for hypertension. The indicated anti-hypertensive medications consisted of beta-blocking agents, angiotensin converting enzyme inhibitors, hydrochlorothiazide, calcium channel blockers and for one subject spironolactone.

Hypertensive African American males and females and white males had somewhat, but not significantly, fewer glomeruli than non-hypertensive subjects of the same gender. Eleven hypertensive white females, however, had significantly fewer glomeruli than 36 non-hypertensive white females ($P=0.02$). Hypertensive subjects had larger glomeruli than their non-hypertensive counterparts among all race and gender groups, and African American males had significantly larger glomeruli than white males ($P<0.001$ for all males, $P=0.04$ for hypertensive males). There were no significant racial differences in the severity of glomerulosclerosis, cortical fibrosis and arteriosclerosis.

For 18- to 70-year-old subjects, a strong inverse correlation was seen between $V_{GLO}$ and $N_{GLO}$ and strong direct correlations were found between $V_{GLO}$, hypertension and BMI (Table 3). Pairwise relationships showed the severity of glomerulosclerosis, cortical fibrosis and arteriosclerosis to be strongly correlated with age and hypertension. Multivariate
Table 1. Subjects dying with hypertension or being identified as hypertensive before age 30

<table>
<thead>
<tr>
<th>Race</th>
<th>Age at death (years)</th>
<th>Gender</th>
<th>BMI (kg/m²)</th>
<th>N_{glomeruli}</th>
<th>MAP (mmHg)</th>
<th>Cause of death</th>
<th>Age (years) at diagnosis of hypertension</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>25</td>
<td>Female</td>
<td>33.1</td>
<td>993 299</td>
<td>115</td>
<td>Postpartum cardiomyopathy</td>
<td>23</td>
</tr>
<tr>
<td>AA</td>
<td>25</td>
<td>Female</td>
<td>50.0</td>
<td>1 066 778</td>
<td>109</td>
<td>Pulmonary embolus</td>
<td>25</td>
</tr>
<tr>
<td>AA</td>
<td>28</td>
<td>Male</td>
<td>41.2</td>
<td>1 163 809</td>
<td>113</td>
<td>Myocardial infarct</td>
<td>28</td>
</tr>
<tr>
<td>AA</td>
<td>28</td>
<td>Male</td>
<td>26.1</td>
<td>1 332 604</td>
<td>112</td>
<td>Rheumatic heart disease</td>
<td>27</td>
</tr>
<tr>
<td>AA</td>
<td>29</td>
<td>Female</td>
<td>25.5</td>
<td>638 228</td>
<td>115</td>
<td>Pulmonary embolus</td>
<td>29</td>
</tr>
<tr>
<td>White</td>
<td>30</td>
<td>Male</td>
<td>33.1</td>
<td>1 208 151</td>
<td>119</td>
<td>Pulmonary embolus</td>
<td>27</td>
</tr>
<tr>
<td>AA</td>
<td>39</td>
<td>Female</td>
<td>24.3</td>
<td>874 539</td>
<td>118</td>
<td>Stroke</td>
<td>25</td>
</tr>
<tr>
<td>AA</td>
<td>42</td>
<td>Male</td>
<td>43.0</td>
<td>1 162 345</td>
<td>113</td>
<td>Myocardial infarct</td>
<td>28</td>
</tr>
<tr>
<td>AA</td>
<td>42</td>
<td>Female</td>
<td>35.8</td>
<td>709 290</td>
<td>112</td>
<td>Stroke</td>
<td>17</td>
</tr>
<tr>
<td>AA</td>
<td>43</td>
<td>Female</td>
<td>22.1</td>
<td>917 875</td>
<td>115</td>
<td>Myocardial infarct</td>
<td>29</td>
</tr>
<tr>
<td>AA</td>
<td>43</td>
<td>Male</td>
<td>29.2</td>
<td>391 733</td>
<td>115</td>
<td>Pulmonary embolus</td>
<td>20</td>
</tr>
<tr>
<td>White</td>
<td>44</td>
<td>Female</td>
<td>26.9</td>
<td>751 245</td>
<td>117</td>
<td>Stroke</td>
<td>28</td>
</tr>
<tr>
<td>AA</td>
<td>46</td>
<td>Male</td>
<td>26.9</td>
<td>1 012 409</td>
<td>120</td>
<td>Myocardial infarct</td>
<td>27</td>
</tr>
<tr>
<td>White</td>
<td>49</td>
<td>Male</td>
<td>30.5</td>
<td>939 893</td>
<td>119</td>
<td>Myocardial infarct</td>
<td>19</td>
</tr>
<tr>
<td>11AA, 3W</td>
<td>7 male, 7 female</td>
<td>32.0 ± 8.1</td>
<td>940 371 ± 252 912</td>
<td>117 ± 6</td>
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AA, African American; N_{glomeruli} number of glomeruli per kidney (right).

least squares and robust regressions were similar for the dependent structural variables of V_{glomeruli}, glomerulosclerosis, cortical fibrosis and arterial intimal thickening when analyzed for the clinical variables of age, race, gender, BMI, hypertension, diabetes, CAD/CVD and hypertension treatment (Table 4). All structural variables were significantly associated with hypertension but not gender. African American race was significantly related to V_{glomeruli} (P < 0.01) and arterial intimal thickening (0.01-0.001) by least squares and robust regression. Diabetes was significantly correlated with cortical fibrosis. The average V_{glomeruli} (9.90 ± 3.72) and percent glomerulosclerosis (6.58 ± 6.14) of diabetics were greater than that of hypertensive non-diabetics (V_{glomeruli} 8.13 ± 2.70, percent glomerulosclerosis 4.98 ± 6.09), but neither difference was significant with the small number of diabetics (V_{glomeruli} P = 0.11, percent glomerulosclerosis P = 0.43). In multivariate least squares but not robust regression, diabetes was significantly related to V_{glomeruli}. The treatment of hypertension was not linked to any of the dependent variables.

The increases in V_{glomeruli} with hypertension were present for both African Americans and whites throughout the range of N_{glomeruli} (Figure 2) and BMI (Figure 3). Figure 4 presents the relationships between V_{glomeruli} and glomerulosclerosis and shows that, compared with non-hypertensives, the increased V_{glomeruli} was found in hypertensive subjects with only mild glomerulosclerosis. With advanced glomerulosclerosis, there was a slight but not significant tendency toward convergence of V_{glomeruli} between hypertensive and non-hypertensive subjects (African Americans non-hypertension, r = 0.105, P = 0.36; hypertension, r = 0.152, P = 0.23; whites non-hypertension, r = 0.089, P = 0.74; hypertension, r = -0.047, P = 0.94).

Effects of race and blood pressure on glomerulosclerosis, arteriosclerosis, cortical fibrosis and V_{glomeruli} among subjects 18-70 years old

In models evaluating race and MAP, a strong direct correlation was found between MAP and arterial intimal thickening, cortical fibrosis and glomerulosclerosis for both races, and a significant increase in V_{glomeruli} with increasing MAP was found for African Americans but not whites (Table 5). A 10 mmHg increase in MAP produced a 0.41 μm² × 10⁶ increase in V_{glomeruli} for African Americans and a 0.21 μm² × 10⁶ increase for...
<table>
<thead>
<tr>
<th></th>
<th>African American</th>
<th>White</th>
<th>P</th>
<th>African American</th>
<th>White</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>Hypertensive, n = 86</td>
<td>Non-hypertensive, n = 61</td>
<td></td>
<td>Hypertensive, n = 44</td>
<td>Non-hypertensive, n = 79</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>46 (40.8–54)</td>
<td>35 (26.5–42)</td>
<td>&lt;0.001</td>
<td>49 (39–54.8)</td>
<td>42 (31–52)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Men</td>
<td>50 (58.1)</td>
<td>27 (44.3)</td>
<td>0.43</td>
<td>33 (75)</td>
<td>43 (54.4)</td>
<td>0.35</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.2 ± 11.4</td>
<td>29.9 ± 7.0</td>
<td>0.41</td>
<td>31.8 ± 10.2</td>
<td>29.3 ± 7.9</td>
<td>0.13</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>119 (114–131)</td>
<td>94 (91–98)</td>
<td>&lt;0.001</td>
<td>113 (113–119)</td>
<td>96 (92–99)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CAD or CVD death</td>
<td>47 (54.7)</td>
<td>13 (21.3)</td>
<td>0.01</td>
<td>56 (8)</td>
<td>15 (19.0)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Diabetes</td>
<td>9 (10.5)</td>
<td>0</td>
<td>0.01</td>
<td>1 (2.3)</td>
<td>1 (1.3)</td>
<td>0.7</td>
</tr>
<tr>
<td>Treated hypertension</td>
<td>42 (48.8)</td>
<td>–</td>
<td></td>
<td>20 (45.5)</td>
<td>–</td>
<td>0.96</td>
</tr>
<tr>
<td>N_{glomeruli}: men</td>
<td>953 ± 350.054</td>
<td>1 021 582 ± 526 581</td>
<td>0.41</td>
<td>946 339 ± 348 970</td>
<td>1 019 957 ± 319 696</td>
<td>0.34</td>
</tr>
<tr>
<td>N_{glomeruli}: women</td>
<td>830 ± 234 445</td>
<td>920 912 ± 213 297</td>
<td>0.10</td>
<td>699 526 ± 184 651</td>
<td>922 265 ± 291 882</td>
<td>0.02</td>
</tr>
<tr>
<td>V_{glomeruli} (µm(^3) × 10^5): men</td>
<td>8.18 (7.13–11.33)</td>
<td>7.04 (5.92–8.28)</td>
<td>0.01</td>
<td>7.45 (5.95–8.89)</td>
<td>5.75 (4.55–7.73)</td>
<td>0.01</td>
</tr>
<tr>
<td>V_{glomeruli} (µm(^3) × 10^5): women</td>
<td>8.92 (5.95–10.05)</td>
<td>5.92 (4.80–8.03)</td>
<td>0.01</td>
<td>8.74 (6.57–9.88)</td>
<td>6.12 (4.62–6.97)</td>
<td>0.01</td>
</tr>
<tr>
<td>Glomerulosclerosis (%)</td>
<td>2.96 (1.29–6.85)</td>
<td>0.96 (0.36–2.38)</td>
<td>&lt;0.001</td>
<td>2.64 (1.60–4.74)</td>
<td>1.03 (0.54–2.48)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cortical fibrosis</td>
<td>0.05 (0.02–0.11)</td>
<td>0.02 (0–0.08)</td>
<td>&lt;0.001</td>
<td>0.05 (0.03–0.08)</td>
<td>0.02 (0.01–0.04)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Arterial intimal thickening</td>
<td>0.11 (0.06–0.18)</td>
<td>0.02 (0–0.08)</td>
<td>&lt;0.001</td>
<td>0.08 (0.04–0.16)</td>
<td>0.03 (0–0.06)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Continuous values expressed as mean ± standard deviation if data are normally distributed, or median (25th–75th percentiles) for non-normally distributed data. Discrete values expressed as number (%).

P for race indicates probabilities of racial differences for hypertension/non-hypertension.

Significant differences are bolded.

BMI, body mass index; MAP, mean arterial pressure; CAD, coronary artery disease; CVD, cerebrovascular disease.
white (Figure 5). Nevertheless, racial interactions with MAP were not significant for any of the variables including \( V_{\text{glomerul}} \) and incremental increases in MAP produced nearly identical increases in glomerulosclerosis, cortical fibrosis, and arterial intimal thickening in both races (Figures 5 and 6).

**DISCUSSION**

The findings demonstrated that age and blood pressure were the principal factors linked to the severity of the microanatomical changes of the kidney that can be subsumed under the term hypertensive nephrosclerosis. The \( V_{\text{glomerul}} \) of African American males was larger than that of white males, and in multivariate analyses, African American race was significantly associated with increased arterial intimal thickening. The findings suggested a racial effect, but it appeared to be related to the proportionately greater number of hypertensive African Americans and their more severe hypertension. Additionally, we showed that \( V_{\text{glomerul}} \) was increased with hypertension and BMI, findings that may correspond to data reported from imaging studies in which many patients with mild-to-moderate nephrosclerosis and metabolic factors contributing to chronic kidney disease have elevated GFR and single nephron filtration rates [7, 12, 15].

Glomerular sclerosis that has been associated with loss of pregomeral vascular resistance was seen in the kidneys of only three subjects in the study and, in these kidneys, occurred with severe arteriosclerotic intimal thickening and equal proportions of glomerular obsolescence. In the other kidneys, glomerular obsolescence was the nearly exclusive pattern of glomerular loss.

The infrequency of glomerular solidification in these autopsies contrasts with the high frequency reported in the AASK biopsies [9, 28]. The latter was a cross-sectional analysis of patients with mild-to-moderate renal insufficiency having average serum creatinine levels of 2.9 ± 0.7 mg/dL and GFR of 51.7 ± 13.6 mL/min/1.73 m². Among these biopsies, there was an average of 42 ± 3% global glomerulosclerosis with African Americans having 50 ± 9%. This compares, in our autopsies, with an average 50 ± 6.1% glomerulosclerosis among hypertensive subjects who died of non-renal causes attributed in the majority of cases to CAD/CVD.

The kidneys in our autopsies were examples of mild-to-moderate nephrosclerosis with the three subjects noted above being severely hypertensive and having features of ‘decompensated nephrosclerosis’ [21]. The discerned patterns of glomerulosclerosis and their association with restrictive preglomerular vascular disease indicate that the progression of hypertensive nephropathy was similar in both races [3, 5, 6, 7]. Glomerular collapse associated with a restrictive afferent arteriolar response rather than segmental glomerulosclerosis is the principal mode of glomerular injury in spontaneously hypertensive rats. This is a widely used experimental model of chronic hypertension in which glomerulosclerosis is attributed to reduced glomerular perfusion [29]. In our observational human studies, glomerular obsolescence with preglomerular arteriosclerosis closely resembled these animal studies.

In the current study, only white women demonstrated a significant relationship between elevated blood pressure and low

---

**Table 3. The study cohort aged 18–70**

<table>
<thead>
<tr>
<th></th>
<th>BMI</th>
<th>( N_{\text{glomerul}} )</th>
<th>( V_{\text{glomerul}} )</th>
<th>GS</th>
<th>CF</th>
<th>It</th>
<th>HTN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>0.005 + 0.93</td>
<td>-0.092 0.13</td>
<td>0.131 0.03</td>
<td>0.605 &lt;0.001</td>
<td>0.431 &lt;0.001</td>
<td>0.527 &lt;0.001</td>
<td>0.348 &lt;0.001</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>0.097 0.11</td>
<td>0.234 &lt;0.001</td>
<td>-0.095 0.11</td>
<td>-0.069 0.25</td>
<td>-0.030 0.61</td>
<td>-0.050 0.41</td>
<td></td>
</tr>
<tr>
<td><strong>N_{\text{glomerul}}</strong></td>
<td>-0.383 &lt;0.001</td>
<td>-0.190 &lt;0.01</td>
<td>-0.144 0.02</td>
<td>-0.091 0.13</td>
<td>-0.132 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>V_{\text{glomerul}}</strong></td>
<td>0.131 0.02</td>
<td>0.218 &lt;0.01</td>
<td>0.190 &lt;0.01</td>
<td>0.360 &lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GS</strong></td>
<td>0.648 &lt;0.001</td>
<td>0.558 &lt;0.001</td>
<td>0.395 &lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CF</strong></td>
<td>0.538 &lt;0.001</td>
<td>0.406 &lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>It</strong></td>
<td>0.532 &lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Spearman rank-order pairwise correlation between age, BMI, \( N_{\text{glomerul}} \), \( V_{\text{glomerul}} \), hypertension and the structural features of hypertensive nephropathy.

Within the cells, the upper number is the Spearman correlation coefficient and the lower number is the P-value. For P < 0.05, there is a significant relationship between the two variables. Variables with significant relationships are indicated in bold.

BML: body mass index; GS: glomerulosclerosis; CF: cortical fibrosis; It: arterial intimal thickening; HTN: hypertension.
Table 4. The study cohort aged 18–70

<table>
<thead>
<tr>
<th></th>
<th>Least squares regression</th>
<th>Robust regression</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{glomer}}$ ($m^3 \times 10^6$)</td>
<td>$V_{\text{glomer}}$ ($m^3 \times 10^6$)</td>
<td>Coefficient</td>
<td>Coefficient</td>
</tr>
<tr>
<td>Age</td>
<td>0.013</td>
<td>0.35</td>
<td>0.014</td>
<td>0.31</td>
</tr>
<tr>
<td>Race</td>
<td>−0.841</td>
<td>0.01</td>
<td>−0.841</td>
<td>0.01</td>
</tr>
<tr>
<td>Gender</td>
<td>−0.449</td>
<td>0.16</td>
<td>−0.273</td>
<td>0.39</td>
</tr>
<tr>
<td>BMI</td>
<td>0.067</td>
<td>$&lt;0.001$</td>
<td>0.063</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.114</td>
<td>$&lt;0.01$</td>
<td>1.303</td>
<td>$0.001$</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1.920</td>
<td>0.02</td>
<td>0.990</td>
<td>0.21</td>
</tr>
<tr>
<td>CAD/CVD</td>
<td>0.229</td>
<td>0.51</td>
<td>0.398</td>
<td>0.37</td>
</tr>
<tr>
<td>Treated hypertension</td>
<td>0.207</td>
<td>0.63</td>
<td>0.012</td>
<td>0.98</td>
</tr>
<tr>
<td>Glomerulosclerosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.150</td>
<td>$&lt;0.001$</td>
<td>0.071</td>
<td>$&lt;0.001$</td>
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<tr>
<td>Race</td>
<td>−1.495</td>
<td>0.01</td>
<td>0.266</td>
<td>0.17</td>
</tr>
<tr>
<td>Gender</td>
<td>0.224</td>
<td>0.68</td>
<td>0.234</td>
<td>0.22</td>
</tr>
<tr>
<td>BMI</td>
<td>−0.047</td>
<td>0.10</td>
<td>−0.015</td>
<td>0.13</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.920</td>
<td>0.01</td>
<td>0.694</td>
<td>0.01</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.777</td>
<td>0.57</td>
<td>1.973</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>CAD/CVD</td>
<td>0.365</td>
<td>0.62</td>
<td>−0.256</td>
<td>0.22</td>
</tr>
<tr>
<td>Treated hypertension</td>
<td>−1.066</td>
<td>0.15</td>
<td>0.003</td>
<td>0.99</td>
</tr>
<tr>
<td>Cortical fibrosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.001</td>
<td>$&lt;0.001$</td>
<td>0.001</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Race</td>
<td>−0.014</td>
<td>0.03</td>
<td>−0.006</td>
<td>0.16</td>
</tr>
<tr>
<td>Gender</td>
<td>0.005</td>
<td>0.44</td>
<td>−0.002</td>
<td>0.64</td>
</tr>
<tr>
<td>BMI</td>
<td>−0.001</td>
<td>0.06</td>
<td>−0.0092</td>
<td>0.18</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.028</td>
<td>$&lt;0.001$</td>
<td>0.017</td>
<td>$0.01$</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.044</td>
<td>$&lt;0.01$</td>
<td>0.034</td>
<td>$0.003$</td>
</tr>
<tr>
<td>CAD/CVD</td>
<td>−0.012</td>
<td>0.07</td>
<td>−0.012</td>
<td>$0.01$</td>
</tr>
<tr>
<td>Treated hypertension</td>
<td>−0.003</td>
<td>0.70</td>
<td>0.004</td>
<td>0.46</td>
</tr>
<tr>
<td>Arterial intimal thickening</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.002</td>
<td>$&lt;0.001$</td>
<td>0.002</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Race</td>
<td>−0.028</td>
<td>0.01</td>
<td>−0.022</td>
<td>$0.001$</td>
</tr>
<tr>
<td>Gender</td>
<td>−0.002</td>
<td>0.82</td>
<td>−0.006</td>
<td>0.39</td>
</tr>
<tr>
<td>BMI</td>
<td>−0.001</td>
<td>0.06</td>
<td>−0.0003</td>
<td>0.36</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.062</td>
<td>$&lt;0.001$</td>
<td>0.040</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Diabetes</td>
<td>−0.037</td>
<td>0.08</td>
<td>−0.019</td>
<td>0.26</td>
</tr>
<tr>
<td>CAD/CVD</td>
<td>0.018</td>
<td>0.15</td>
<td>0.013</td>
<td>0.08</td>
</tr>
<tr>
<td>Treated hypertension</td>
<td>−0.027</td>
<td>0.31</td>
<td>−0.007</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Multivariate analysis by least squares and robust regression showing the relative contributions of the clinical factors of age, race, gender, BMI, hypertension, diabetes, CAD/CVD death, and treatment of hypertension to $V_{\text{glomer}}$ glomerulosclerosis, cortical fibrosis and arterial intimal thickening.

Assigned scores: male = 1, female = 2; African American race = 1, white race = 2. Hypertension = 1, diabetes = 1, CAD/CVD = 1 and treated hypertension = 1 (all verses 0).

Significant relationships are bolded.
glomerular number. This may indicate that low $N_{\text{glo}}$ plays a role in the hypertension of white women, or it may be a false association related to the small number of 11 white hypertensive women in our sample. Low nephron number was not seen in individuals identified as being hypertensive or dying with hypertension before age 30 who might be anticipated to have a predisposing nephron deficit. The findings generally contradict the hypothesis of essential hypertension being related to low nephron number proposed by Keller et al. [30].

Keller et al. [30] described two distinct groups of subjects in which 10 hypertensives had a range of 531,140–954,893 glomeruli and 10 non-hypertensives a range of 884,458–1,959,914 glomeruli. The centile distribution of $N_{\text{glo}}$ for their hypertensive subjects is essentially the same that we have reported for hypertension among both African Americans and whites [19, 20]. But the median $N_{\text{glo}}$ for their non-hypertensives is above the 75th percentile for our non-hypertensive subjects and for other reported distributions of glomerular number in the human kidney [18, 24, 25, 30, 31]. They seem to have found a small group of subjects in which very high nephron number protects against hypertension [20]. In our autopsy sample, the mildly reduced number of glomeruli in hypertensive subjects is more compatible with a model in which lower $N_{\text{glo}}$ is the result of glomerular loss owing to obsolescence.

The principal determinant of adult $V_{\text{glo}}$ is an apparently innate inverse relationship to $N_{\text{glo}}$, in which low glomerular number is associated with large glomerular volume [18, 24, 25, 30, 32]. BMI and hypertension are correlated with increased $V_{\text{glo}}$ independently of each other and of $N_{\text{glo}}$ [24, 25, 32]. In this regard, glomerular hypertrophy acts in a dependent manner to hypertension and as having developmental associations with glomerulosclerosis and arterial intimal thickening.
The relationships suggest that the enlarged glomeruli may be susceptible to collapse and obsolescence when there is a reduction in glomerular blood flow [29].

Our methods and findings resemble those of Kaslak [33] in his analysis of atherosclerotic vascular disease and age-associated changes of the kidney in autopsies from the Hennepin County Medical Examiner's office. In pairwise regression, mean glomerular area, intrarenal arterial wall thickening, and percent glomerulosclerosis were significantly correlated with age and hypertension; whereas in multivariate regression, glomerular area was related to atherosclerotic CAD but not glomerulosclerosis. This is similar to the associations that we find between glomerulosclerosis and glomerular volume in which increased $V_{glo}$ correlated with hypertension. In neither study was glomerulosclerosis associated with a compensatory increase in glomerular size [33]. The treatment of hypertension was also evaluated in both studies with no effect on glomerular size being demonstrated [33]. Hypertension and CAD/CVD as well as treatment for hypertension are closely linked phenomena, and independent associations with any particular manifestation of hypertension-associated disease may be difficult to find; nonetheless, the absence of any relationship between glomerulosclerosis and average glomerular size brings into question whether a compensatory increase in $V_{glo}$ actually occurs in kidneys with age-related glomerular loss.

The increased African American risk for non-diabetic kidney disease including FSGS and hypertension has a genetic component related to apolipoprotein L1 gene (APOL1) sequence variants. Among African Americans, subjects with one APOL1 risk allele are found to have an OR of 1.26 and those with two risk alleles an OR of 7.3 for hypertension-associated ESRD that has been related to a susceptibility for

---

**Table 5. The study cohort aged 18–70**

<table>
<thead>
<tr>
<th></th>
<th>MAP Coef (P)</th>
<th>Interaction Coef (P)</th>
<th>Model $r^2$ (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>African American</td>
<td>White</td>
<td></td>
</tr>
<tr>
<td>$V_{glo}$ (um$^2 \times 10^6$)</td>
<td>0.042 (&lt;0.01)</td>
<td>0.023 (0.43)</td>
<td>-0.635 (0.13)</td>
</tr>
<tr>
<td>Glomerulosclerosis</td>
<td>0.126 (&lt;0.001)</td>
<td>0.082 (&lt;0.001)</td>
<td>-0.914 (0.16)</td>
</tr>
<tr>
<td>Cortical fibrosis</td>
<td>0.001 (&lt;0.001)</td>
<td>0.002 (&lt;0.001)</td>
<td>-0.005 (0.52)</td>
</tr>
<tr>
<td>Intimal thickening</td>
<td>0.003 (&lt;0.001)</td>
<td>0.003 (&lt;0.001)</td>
<td>-0.007 (0.54)</td>
</tr>
</tbody>
</table>

Multivariate least squares regression showing the relationships of race, MAP and their interactions with the pathological features of hypertensive nephropathy. Coefficients are presented for the non-interactive effects of MAP for $V_{glo}$ glomerulosclerosis (%), cortical fibrosis, and arterial intimal thickening and for the MAP race interactions. Model $r^2$ is adjusted and reflects interactions. MAP, mean arterial pressure (mmHg); Coef, coefficient; P, probability.
glomerulosclerosis [4, 34]. We are currently investigating the relationships between APOL1 risk alleles and the structural features of hypertensive nephropathy. The initial results show a strong linkage to hypertension but also suggest that APOL1 risk alleles may be associated with excessive glomerular enlargement [35].

In summary, we show that the setting of mild-to-moderate nephrosclerosis, glomerular hypertrophy is a primary attribute of hypertensive nephropathy. We suggest that glomerular hypertrophy precedes the later glomerular collapse that leads to glomerular obsolescence through a mechanism that simultaneously promotes preglomerular arteriosclerosis. Our choice of independent and dependent variables was based upon the current physiological concepts of essential hypertension being the result of abnormal pressure natriuresis or oxidative stress and the structural changes of nephrosclerosis, particularly the vasculopathies, being secondary to the elevation of blood pressure [5, 36, 37]. Many investigators in this field, however, have proposed that hypertension is secondary to age-related vascular changes [5, 6, 38]. The current and similar descriptive and observational studies are not designed to evaluate the causality of clinical and structural relationships, and the strength of the associations between blood pressure and nephrosclerosis make it difficult to separate any effect of race. It is anticipated that the continuing genetic studies may clarify a racial effect on glomerular and vascular structure and possibly a link to blood pressure [3].
ACKNOWLEDGEMENTS

This research was funded by grants from the National Institutes of Health (NIH I RO1 DK065970-01), NIH Center of Excellence in Minority Health (SP20-M005534-02), the National Medical Research Council of Australia, Janssen-Cilag Australia Pty Ltd and the American Heart Association (Southeastern Affiliate).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicting interests and no financial disclosures. The results presented in this paper have not been published previously in whole or part, except in abstract format.

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38. Tracy RE. Renovascular pathologies of hypertension and the rise of blood pressure with age in blacks and whites. Semin Nephrol 1996; 16: 126–133

Received for publication: 21.7.2013; Accepted in revised form: 27.10.2013
Chapter 4

Glomerular hypertrophy in subjects with low nephron number: contributions of sex, body size and race

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Design of experiment, data collection, analysis, writing and editing of multiple drafts</td>
<td>85</td>
</tr>
</tbody>
</table>

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%) for student co-authors only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Douglas-Denton, RN</td>
<td>Data collection</td>
<td></td>
</tr>
<tr>
<td>Zimanyi, MA</td>
<td>Data collection</td>
<td></td>
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<tr>
<td>Armitage, JA</td>
<td>Experimental design</td>
<td></td>
</tr>
<tr>
<td>Hughson, MD</td>
<td>Tissue collection and editing of multiple drafts</td>
<td></td>
</tr>
<tr>
<td>Kerr, PG</td>
<td>Editing of multiple drafts</td>
<td></td>
</tr>
<tr>
<td>Bertram, JF</td>
<td>Editing of multiple drafts</td>
<td></td>
</tr>
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</table>

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate’s and co-authors’ contributions to this work.

<table>
<thead>
<tr>
<th>Candidate’s Signature</th>
<th>Date: 21-5-14</th>
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<table>
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<th>Date: 21-5-14</th>
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</table>
Glomerular hypertrophy in subjects with low nephron number: contributions of sex, body size and race

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ABSTRACT

Background. We have shown that low nephron number (Nglomer) is a strong determinant of individual glomerular volume (IGV) in male Americans. However, whether the same pattern is present in female Americans remains unclear. The contributions of body surface area (BSA) and race to IGV in the context of Nglomer also require further evaluation.

Methods. Kidneys without overt renal disease were collected at autopsy in Mississippi, USA. The extremes of female Nglomer were used to define high and low Nglomer for both sexes. Nglomer and IGV were estimated by design-based stereology. A total of 24 African and Caucasian American females (n = 12 per race; 6 per Nglomer extreme) were included. These subjects were subsequently matched to 24 comparable males by age and Nglomer and to 18 additional males by age, Nglomer and BSA.

Results. IGV average and variance were very similar in female African and Caucasian Americans with high and low Nglomer. Males with low Nglomer from both races showed greater IGV average and variance than comparable females matched by age and Nglomer. These differences in IGV between sexes were not observed in Caucasian Americans with low Nglomer that were matched by age, Nglomer and BSA. In contrast, glomeruli from African Americans were larger than those from Caucasian Americans, especially in subjects with high Nglomer.

Conclusions. While female Americans with low Nglomer did not show glomerular hypertrophy, comparable males with low Nglomer showed marked glomerular hypertrophy that was closely associated with high BSA. Glomerular size in African Americans may be confounded by multiple additional factors.

Keywords: body size, glomerular hypertrophy, nephron number, race, sex

INTRODUCTION

Chronic kidney disease (CKD) is a major health problem worldwide [1, 2]. To date, early detection based on screening of high risk patients and effective early intervention are the best approaches to delay CKD development and progression [3]. It has been suggested that subjects with low total nephron number (Nglomer) may be at greater risk of kidney disease [4-8]. According to the hyperfiltration theory [9], low Nglomer—whether congenital or acquired—results in compensatory hypertrophy of remaining glomeruli in order to match physiological requirements and sustain renal function. Initially, glomerular hypertrophy appears to be an appropriate compensatory response. However, there is a point at which hypertrophy is unsustainable, leading to glomerulosclerosis, proteinuria and reduced renal function [5]. Interestingly, glomerular hypertrophy has pivotal roles in the development of focal and segmental glomerulosclerosis [10, 11] and the progression of diabetic nephropathy [12-14], two of the most common causes of CKD. While the boundary between adaptive and pathological glomerular hypertrophy remains unclear, efforts to identify risk factors associated with glomerular hypertrophy in subjects
without renal disease are essential to characterize this process [15] and develop preventive strategies in the future.

The role of sex in the development and progression of CKD has been widely debated in recent years [16]. A comprehensive meta-analysis by Neugarten et al. [17] concluded that renal disease in women progressed at a slower rate than in men. There is also evidence from several animal models of renal disease, such as aging, renal ablation, hypertension and polycystic kidney disease, in which females are relatively protected from the development of renal pathology compared with males [18].

It has been proposed that structural factors (i.e. $N_{glomer}$) could explain this relative protection observed in females [19], but so far all findings have failed to support this hypothesis. Our group has reported that if anything, American females have slightly lower $N_{glomer}$ than males [20] and Neugarten et al. [21] showed that despite having similar $N_{glomer}$ between sexes, Danish males had larger glomeruli than females, which was not related to sex and was closely associated with body surface area (BSA), a measure of body size and surrogate marker of renal cortical volume [22, 23]. In multiple autopsy studies, we have consistently shown larger BSA in males than in female Americans [20, 24, 25], which provides a unique opportunity to explore the contribution of BSA to glomerular size.

There are several methods to investigate glomerular size in human kidneys [4]. For example, mean glomerular volume ($V_{glomer}$) provides a single summary statistic for all glomeruli in the kidney [26, 27]. We have recently been reporting individual glomerular volume (IGV) [28], which provides new insights into glomerular size variability within kidneys. Zimanyi et al. [29] reported that Caucasian American males with low $N_{glomer}$ had greater average IGV and more heterogeneous IGV distributions than comparable subjects with high $N_{glomer}$, both of which suggested a process of glomerular hypertrophy. Moreover, this study [29] also showed that African American males presented glomerular hypertrophy, even in subjects with robust $N_{glomer}$. However, it remains unclear if these trends could also be found in females.

The current study provides a unique approach to further understand the role of human compensatory glomerular hypertrophy in pre-disease stages. We hypothesized that people with low $N_{glomer}$ will show glomerular hypertrophy when physiological demands (represented by BSA) cannot be met by the existing number of nephrons (represented by $N_{glomer}$); a scenario that is characterized by a combination of low $N_{glomer}$ and high BSA. Two aims were defined: (i) to evaluate for the first time IGV estimates between extremes of $N_{glomer}$ in female African and Caucasian Americans, a cohort with innate low BSA; and (ii) to compare IGV distributions between sexes in the context of $N_{glomer}$ BSA and race.

MATERIALS AND METHODS

Tissue collection and subject selection
Kidneys were collected during autopsies performed at the University of Mississippi Medical Center, Jackson, MS, USA [24, 25] with the approval of the Institutional Review Board of the University of Mississippi Medical Center and the Monash University Human Research Ethics Committee. Subjects were included if their kidneys were of approximately equal size and showed grossly normal or mild-to-moderate arteriolonephrosclerosis with a uniform granular subcapsular cortex. Kidneys from patients with diabetes were analysed if they showed mild-to-moderate arteriolonephrosclerosis but were excluded if there was microscopic evidence of diabetic nephropathy. Based on this, two diabetic Caucasian Americans were included in this study. Furthermore, kidneys were excluded if coarse pits or any angular or depressed cortical scars were found. Kidneys from patients who had clinically diagnosed kidney disease and/or diagnosed congenital abnormalities (i.e. congenital heart disease) were also excluded.

Extremes of $N_{glomer}$ were defined based on the best possible pairing between extreme quintiles within 77 African Americans and 52 Caucasian Americans. High $N_{glomer}$ was defined as $\geq 1.0$ million glomeruli and low $N_{glomer}$ as $\leq 0.66$ million glomeruli. A total of 24 females, including 12 African and 12 Caucasian Americans (6 with high and 6 with low $N_{glomer}$) were selected for analysis. These subjects were carefully age matched within each race and represented our matching reference group for the rest of the study. Next, we analysed IGV average and variance between comparable females and males. Because the extremes of $N_{glomer}$ distributions were different between sexes, we were not able to compare strict extremes of $N_{glomer}$. Therefore, males were matched to females within each race only by age and $N_{glomer}$ a matching strategy referred to as model 1. To account for differences in BSA between sexes, we selected additional males and matched them to females within each race by age, $N_{glomer}$ and BSA; a matching strategy referred to as model 2. Multiple selection criteria for models 1 and 2 did not allow a higher sample size per group. A total of 66 subjects were included in this study (n = 32 for African Americans and n = 34 for Caucasian Americans), including all females and males.

Demographic data

General demographic data, including age, race, sex, height, body weight, medical history (i.e. history of diabetes or hypertension), blood pressure (BP) and medical treatment (i.e. prescribed anti-hypertensive medication) were all obtained from the medical records and forensic reports. Body mass index (BMI) was calculated based on weight and height [30] at the time of autopsy. BSA was calculated using the Mosteller equation [31]. BP from terminal hospital admissions was not used unless patients were diagnosed as hypertensive and BP was consistently elevated. Mean arterial blood pressure (MAP) was calculated from an average of at least three blood pressure measurements and was only available for 45 patients. Hypertension status was defined as hypertensive or normotensive based on pathology findings (i.e. presence of cardiomegaly and severity of intracranial arteriosclerosis), and medical records (i.e. history of hypertension and consistently elevated BP $\geq 140/90$ mmHg) [20, 25, 32].

Design-based stereology

Design-based stereology was performed at Monash University. $N_{glomer}$ was estimated in all 66 subjects as described previously [20, 24–26, 33–35] applying a combination of
systematic uniform random sampling and the physical director/fractionator combination. Some of the male IGV values have previously been published [15, 28, 29, 36–38].

Formalin-fixed tissue from female kidneys (n = 24) stored at Monash University was used for glomerular sampling. Medullary kidney slices of 1 cm × 1 cm × 1 mm were embedded in glycol methacrylate (Technovit 7100, Heraeus Kulzer, Germany) and serially sectioned at 10 μm. Every second section was stained with periodic acid Schiff (PAS). Thirty glomeruli per subject were sampled (10 from each cortical zone: superficial, middle and juxtamedullary) using the dissector principle [39]. IGV was calculated using the Cavalieri’s estimator [40]. In short, virtual images were obtained with an Olympus Dorslide system (Olympus Soft Imaging, Tokyo, Japan) equipped with a 20x objective lens and a monochrome slide Peltier cooled digital camera at Monash Micro Imaging. The areas of glomerular tufts were measured by point counting using an orthogonal grid placed over each glomerular profile. Grid size and total magnification were calibrated before each measurement and adjusted to obtain at least 100 grid points per glomerulus [28].

Statistical analysis

Data were analysed using GraphPad Prism version 5.04 for Windows (La Jolla, CA, USA) and Stata version 12.1 (Statistical Software; College Station, TX, USA: StataCorp LP). Values are expressed as median ± interquartile range (IQR) unless otherwise stated. In order to take into account variability within and between subjects, we applied a mixed-effects regression analysis to compare IGV between groups. To reduce potential bias, given the small sample sizes, multi-level models employed restricted maximum likelihood estimates of variance. Multiple regression analysis was also conducted to assess predictors of mean IGV such as race, sex, BSA, BMI, Nglomer = hypertension status, MAP and anti-hypertensive treatment. A logistic regression analysis was applied to assess predictors of hypertensive status, including age, race, sex, age, BSA and Nglomer. Associations between two variables were tested by the Spearman rank coefficient and Kruskal–Wallis test, with Dunn’s post hoc test applied for multiple comparisons. A probability (P) less than 0.05 was considered statistically significant.

RESULTS

General demographics in female subjects

Table 1 shows general demographic data for the age-matched female subjects (matched within a maximum of 6 years), including age, BSA, BMI, hypertensive status and Nglomer. While median age was 38 years for African Americans, it was 45.5 years for Caucasian Americans (P < 0.05). On the basis of BMI, 25% of African American females were obese and 50% were overweight, while 25% of Caucasian American females were obese, but only 17% were overweight. Overall, BSA was higher in African Americans than Caucasian Americans in combined data from both Nglomer groups within each race (P = 0.01). Among females with low Nglomer, the frequency of hypertensive subjects was 67% for African Americans and 33% for Caucasian Americans.

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Table 1. Age-matched female subject pairs in African and Caucasian Americans

<table>
<thead>
<tr>
<th>Pairs</th>
<th>High Nglomer</th>
<th>Low Nglomer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age (years)</td>
<td>BSA (m²)</td>
</tr>
<tr>
<td></td>
<td>African Americans (n = 12)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>23</td>
<td>3.19</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>2.2</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>1.68</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>1.88</td>
</tr>
<tr>
<td>5</td>
<td>49</td>
<td>1.73</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>2.14</td>
</tr>
<tr>
<td>Median</td>
<td>38</td>
<td>2.91</td>
</tr>
<tr>
<td>(RQR)</td>
<td>(28–49)</td>
<td>(1.7–2.2)</td>
</tr>
<tr>
<td></td>
<td>Caucasian Americans (n = 12)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>1.67</td>
</tr>
<tr>
<td>2</td>
<td>41</td>
<td>1.76</td>
</tr>
<tr>
<td>3</td>
<td>44</td>
<td>1.85</td>
</tr>
<tr>
<td>4</td>
<td>47</td>
<td>1.77</td>
</tr>
<tr>
<td>5</td>
<td>47</td>
<td>1.71</td>
</tr>
<tr>
<td>6</td>
<td>59</td>
<td>1.88</td>
</tr>
<tr>
<td>Median</td>
<td>45.5</td>
<td>1.76</td>
</tr>
<tr>
<td>(RQR)</td>
<td>(38–50)</td>
<td>(1.7–1.9)</td>
</tr>
</tbody>
</table>

Nglomer: total nephron number; BSA: body surface area; BMI: body mass index; Pairs: refer to age-matched pairs formed within each race and between Nglomer categories; RQR: interquartile range.

*P < 0.001; **P < 0.01; ***NS: not statistically significant—for comparisons within each race and between Nglomer categories labels were placed in the Low Nglomer column.
First report of individual glomerular volume in female Americans

IGV was highly variable among females, with an 8.2-fold range and a 4.8-fold range between the smallest and largest glomeruli in African and Caucasian Americans, respectively. Importantly, average IGV and variance were very similar between females with high and low \( N_{\text{glom}} \) in both African Americans (\( P = 0.36 \) for average and \( P = 0.26 \) for variance) and Caucasian Americans (\( P = 0.35 \) for average and \( P = 0.13 \) for variance).

General demographics for comparisons between sexes

General demographics for female and male groups are provided in Table 2. Briefly, models 1 and 2 (males) were both comparable to females in terms of age and \( N_{\text{glom}} \). From the total of 66 subjects included in this study, 24 (36%) were defined as hypertensive. From this group, 17 (71%) subjects had a history of prescribed anti-hypertensive medications. Furthermore, hypertensive subjects were more common in groups defined by low \( N_{\text{glom}} \) in both races and sex, especially in African Americans, 67% of whom were hypertensive. Details about the proportion and percentage of subjects with a history of prescribed anti-hypertensive medications by sex and race are provided in Table 2.

Body dimensions

Table 3 provides an overview of available measurements of body dimensions, including height, weight, BSA and BMI. Importantly, BSA and BMI were strongly associated (\( R = 0.83, P < 0.001 \)).

In model 1, males were taller, heavier and showed higher BSA than females from both races and \( N_{\text{glom}} \) categories. However, BMI was very similar between males and females from model 1 in all comparisons, except for Caucasian Americans with high \( N_{\text{glom}} \) who showed a significantly greater BMI than females. Among African Americans, males from model 2 had very similar height, weight, BSA and BMI than females, but they also showed similar height as males from model 1 (\( P > 0.05 \)). More importantly, these subjects presented significantly lower weight, BSA and BMI than females from model 1. In contrast, among African Americans, differences in height, weight, BSA and BMI between males from models 1 and 2 were not sufficient to achieve statistical significance, except in males from model 2 with high \( N_{\text{glom}} \) who showed a significantly reduction in BSA compared with males from model 1.

Comparison of individual glomerular volume between sexes

Model 1: males matched to females by age and \( N_{\text{glom}} \). IGV was remarkably variable among males from model 1, with a 7.7-fold range and an 11.2-fold range between the smallest and largest glomeruli in African and Caucasian Americans, respectively. Both male African Americans with low \( N_{\text{glom}} \) (Figure 1A; \( P = 0.002 \)) and male Caucasian Americans with low \( N_{\text{glom}} \) (Figure 1B; \( P < 0.001 \)) showed greater average IGV than those with high \( N_{\text{glom}} \). Table 4 shows that average IGV was similar between sexes in subjects with high \( N_{\text{glom}} \). In contrast, in subjects with low \( N_{\text{glom}} \), average IGV was significantly higher in males (\( P < 0.01 \)) for African Americans and \( P > 0.001 \) for Caucasian Americans.

Among subjects with high \( N_{\text{glom}} \), IGV variance was similar between sexes in Caucasian Americans (\( P = 0.81 \)), but it was higher in African American males compared with their respective female counterparts (\( P = 0.03 \)). In contrast, among subjects with low \( N_{\text{glom}} \), males showed greater IGV variance than females in both racial groups (\( P = 0.07 \) for African Americans and \( P = 0.008 \) for Caucasian Americans).

Model 2: males matched to females by age, \( N_{\text{glom}} \) and BSA. IGV was also very variable among males from model 2, with a 6.3-fold range and an 8-fold range between the smallest and largest glomeruli in African and Caucasian American females, respectively.

Interestingly, males with high \( N_{\text{glom}} \) had similar average IGV as those with low \( N_{\text{glom}} \) in both African (Figure 2A; \( P = 0.58 \)) and Caucasian Americans (Figure 2B; \( P = 0.40 \)). Table 4 shows that average IGV was also similar between sexes in both races (\( P > 0.05 \) in all comparisons).

IGV variance was also similar between sexes in all categories from Caucasian Americans (\( P > 0.05 \)) and African Americans with high \( N_{\text{glom}} \) (\( P = 0.18 \)). In African Americans with low \( N_{\text{glom}} \), males showed greater IGV variance compared with their respective female pairs (\( P = 0.03 \)).

Comparison of individual glomerular volume between races

Distributions of IGV from African Americans showed considerable overlap between most groups (Figure 3A). Interestingly, distributions of IGV for Caucasian Americans were remarkably similar in all categories, except for males with a combination of low \( N_{\text{glom}} \) and high BSA (model 1), for whom the distribution of IGV was markedly right-shifted (Figure 3B).

Male African Americans with high \( N_{\text{glom}} \) showed average IGV that was higher than that in Caucasian Americans, including 52% for model 1 (\( P < 0.001 \)) with significant interactions from age (\( P < 0.01 \)) and \( N_{\text{glom}} \) (\( P < 0.03 \)), and 45% for model 2 (\( P = 0.11 \)) without any interactions with age, \( N_{\text{glom}} \) or BSA. Among males with low \( N_{\text{glom}} \), average IGV was very similar between races in model 1 (\( P = 0.30 \)) and model 2 (\( P = 0.21 \)), but IGV variance was higher in African Americans from model 2 compared with their respective Caucasian counterparts (\( P = 0.03 \)).

What is the contribution of hypertension?

A multiple regression model was applied, including mean IGV per subject as the outcome and BSA, \( N_{\text{glom}} \) race and hypertensive status as independent variables (\( F = 8.82, R^2 = 0.35, P < 0.001 \)). While African American race (\( R = 0.26, P < 0.05 \)), BSA (\( R = 0.31, P < 0.01 \)) and \( N_{\text{glom}} \) (\( R = 0.37, P < 0.01 \)) all predicted increases in mean IGV per subject, the contribution of hypertensive status was not significant (\( R = 0.15, P > 0.05 \)). When MAP was introduced in this model (\( F = 4.87, R^2 = 0.30, P < 0.01 \)), the pattern was the same, with strong contributions.
### Table 2. Analysis of general demographics by sex and Nxglomeruli in African and Caucasian Americans

<table>
<thead>
<tr>
<th></th>
<th>Females</th>
<th>Model 1 for males (matched to females by age and Nxglomeruli)</th>
<th>Model 2 for males (matched to females by age, Nxglomeruli and BSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age (years)</td>
<td>Nxglomeruli (million)</td>
<td>HTN (proportion, %)</td>
</tr>
<tr>
<td>African Americans</td>
<td>High</td>
<td>38 (28-49)</td>
<td>1.16</td>
</tr>
<tr>
<td>(n = 32)</td>
<td>Low</td>
<td>36.5 (30-45)</td>
<td>0.59</td>
</tr>
<tr>
<td>Caucasian Americans</td>
<td>High</td>
<td>45.5 (38-50)</td>
<td>1.1</td>
</tr>
<tr>
<td>(n = 34)</td>
<td>Low</td>
<td>46.5 (39-55)</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Values for age and Nxglomeruli are expressed as median (inter quartile range). HTN (HTN: hypertensive status) is expressed as a proportion and percentage of hypertensive subjects; Rx HTN (history of prescribed antihypertensives medication), which is expressed as a proportion and percentage of subjects receiving antihypertensive therapy to control blood pressure; Nxglomeruli total nephron number. **P < 0.05; all statistical comparisons were performed between females and model 1 for males and between females and model 2 for males; NA, not applicable.

### Table 3. Analysis of body dimensions by sex and Nxglomeruli in African and Caucasian Americans

<table>
<thead>
<tr>
<th></th>
<th>Females</th>
<th>Model 1 for males (matched to females by age and Nxglomeruli)</th>
<th>Model 2 for males (matched to females by age, Nxglomeruli and BSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height (cm)</td>
<td>Weight (Kg)</td>
<td>BMI (Kg/m²)</td>
</tr>
<tr>
<td>African Americans</td>
<td>High</td>
<td>165 (156-175)</td>
<td>68 (56-101)</td>
</tr>
<tr>
<td>(n = 32)</td>
<td>Low</td>
<td>165 (159-173)</td>
<td>67</td>
</tr>
<tr>
<td>Caucasian Americans</td>
<td>High</td>
<td>165 (157-169)</td>
<td>67 (53-77)</td>
</tr>
<tr>
<td>(n = 34)</td>
<td>Low</td>
<td>159 (157-163)</td>
<td>62</td>
</tr>
</tbody>
</table>

Values are expressed as median (inter quartile range). BMI, body mass index; BSA, body surface area; Rx HTN, history of prescribed antihypertensives medication. **P < 0.05; all statistical comparisons were performed between females and model 1 for males and between females and model 2 for males; NA, not applicable.
FIGURE 1: Sex comparisons of individual glomerular volume (IGV) by total nephron number ($N_{glomer}$) in African and Caucasian Americans. Males were matched to females based on age and $N_{glomer}$ only (model 1). Each circle represents one glomerulus and each column represents aggregated data from 6 subjects per category (30 glomeruli per subject); black circles for African Americans, white circles for Caucasian Americans; grey and black lines represent mean IGV and standard deviation per category.

### Table 4. Summary of individual glomerular volume (IGV) values; sex comparisons in African and Caucasian Americans

<table>
<thead>
<tr>
<th></th>
<th>Females</th>
<th>Model 1 for males matched to females by age and $N_{glomer}$</th>
<th>Model 2 for males matched to females by age, $N_{glomer}$, and BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Smallest</td>
<td>Largest</td>
<td>Mean IGV (SD) [μm$^3$]</td>
</tr>
<tr>
<td>African Americans (n = 32)</td>
<td>High $N_{glomer}$</td>
<td>2.13</td>
<td>8.11</td>
</tr>
<tr>
<td>Low $N_{glomer}$</td>
<td>0.99</td>
<td>7.49</td>
<td>4.65</td>
</tr>
<tr>
<td>Caucasian Americans (n = 34)</td>
<td>High $N_{glomer}$</td>
<td>1.62</td>
<td>6.76</td>
</tr>
<tr>
<td>Low $N_{glomer}$</td>
<td>1.46</td>
<td>6.99</td>
<td>4.07</td>
</tr>
</tbody>
</table>

$N_{glomer}$: extremes: High or Low based on extremes of the female $N_{glomer}$ distribution. Mean IGV represents the mean value of all measured glomeruli per group (30 glomeruli per subject, 6 subjects per group). SD: standard deviation. NS: not statistically significant.

***P < 0.001 and **P < 0.01 in bold. #P > 0.05; all statistical comparisons were performed between females and model 1 for males and between females and model 2 for males.

FIGURE 2: Sex comparison of individual glomerular volume (IGV) by total nephron number ($N_{glomer}$) in African and Caucasian Americans in the context of body surface area (BSA). Males were matched to females by age, $N_{glomer}$ and BSA (model 2). Each circle represents one glomerulus and each column represents aggregated data from 6 subjects per category (30 glomeruli per subject). Grey and black lines represent mean IGV and standard deviation per category.
from African American race ($\beta = 0.34$, $P < 0.05$), BSA ($\beta = 0.28$; $P < 0.01$) and $N_{\text{glomer}}$ ($\beta = -0.41$; $P < 0.01$), but no contribution from MAP ($\beta = 0.01, P = 0.94$). When anti-hypertensive therapy was introduced in this model ($F = 8.30, R^2 = 0.23, P < 0.0001$), the same pattern was also confirmed with strong contributions from African American race ($\beta = 0.25$, $P < 0.05$), BSA ($\beta = 0.31, P < 0.01$) and $N_{\text{glomer}}$ ($\beta = -0.36, P < 0.01$), but no significant contribution from anti-hypertensive therapy ($\beta = 0.17, P > 0.05$). Furthermore, these models were applied again, this time using BMI instead of BSA. In this case, BMI, $N_{\text{glomer}}$ and African American race were the best predictors of mean IGV ($P < 0.03$) with no significant contributions from hypertension and anti-hypertensive therapy ($P > 0.05$).

A logistic regression using hypertensive status as the outcome variable with race, sex, age, BSA and $N_{\text{glomer}}$ as independent variables showed that neither the model, nor the variables were able to predict hypertension (data not shown). However, using a Spearman rank coefficient analysis, hypertensive status showed a strong positive association with age ($R = 0.36, P < 0.01$) and a weaker inverse correlation with $N_{\text{glomer}}$ ($R = -0.29, P < 0.05$).

**DISCUSSION**

The present study has three major findings: (i) American females with low $N_{\text{glomer}}$ did not show glomerular hypertrophy; (ii) American males with low $N_{\text{glomer}}$ showed marked glomerular hypertrophy that was closely associated with large body size; and (iii) glomerular size in African Americans may be confounded by multiple additional factors.

There is evidence of increases in glomerular size under physiological conditions, for example normal body growth [41, 42] and in association with multiple CKD risk factors [15, 28, 29, 34, 36–38]. It has been suggested that subjects with low $N_{\text{glomer}}$ may be at greater risk of kidney disease [4–8], especially because low $N_{\text{glomer}}$ is a strong driver of glomerular hypertrophy [26, 27]. According to the hyperfiltration theory [43], a setting of low $N_{\text{glomer}}$ may require a compensatory step in order to sustain renal function. Since human glomerulogenesis does not continue in the postnatal period [44], glomerular hypertrophy becomes a necessary compensatory step to meet physiological demands. However, humans have a substantial functional reserve [45], a protective factor against nephron under-endowment or nephron loss, which suggests that glomerular hypertrophy may be required if an additional stimulus is present.

In the clinical setting, parameters such as body weight [46], BSA [47] and BMI [48, 49] may represent the metabolic demands of the potential kidney recipient. Our current study suggests that high metabolic demands coupled with low $N_{\text{glomer}}$ are closely associated with glomerular hypertrophy. We propose that low $N_{\text{glomer}}$ is part of a bigger problem, which is the preservation of a proper balance between filtration surface area and physiologic demands. Indirect clinical confirmation of this hypothesis is supported by transplantation data [50, 51]. Oh et al. [50] provided direct evidence of an effect of donor graft mass and recipient metabolic demands on early graft function. This was confirmed by Tent et al. [51] who found that transplanted kidneys adapt to the recipient’s body size and demands, independent of sex, without a deleterious effect in renal function and outcome up to mid-term long.

Several studies in humans have shown no differences in glomerular size between sexes [52–54]. However, differences in methodology and race across these studies may confound the interpretation. Neugarten et al. [21] reported that despite similar $N_{\text{glomer}}$ between sexes, males had larger glomeruli than females, which was closely associated with BSA rather than sex. While both findings were confirmed by the current study, we added a new component: glomerular size variability. As we described in males [29], glomerular size also varies significantly in female Americans and it appears to be exacerbated by glomerular hyperfiltration.
stressors (i.e. low $N_{glomer}$). Several theories have been proposed for this variability in the normal human kidney based around the concepts of glomerular hyper/hypo perfusion, including vascular changes that affect blood flow delivery to certain areas of the renal cortex [55] or specific glomerular features (i.e. abular glomerulitis) [56]. The question as to why some glomeruli undergo hypertrophy and others do not still remains and merits further assessment in future studies.

Low $N_{glomer}$ is a CKD risk factor that is currently difficult to use in clinical practice [4, 5, 8, 27]. This is primarily because of the lack of non-invasive methods for estimating $N_{glomer}$ in humans. The gold-standard method for estimating $N_{glomer}$ is based on unbiased stereology, which is by definition invasive. Interestingly, the first steps towards the development of non-invasive magnetic resonance imaging to quantify both glomerular number and size have recently been reported in ex vivo rat kidneys [57, 58], and initial efforts towards in vivo analysis of glomerular number and volume have commenced [59] and have the potential for the design and execution of future longitudinal studies.

At present, clinicians can use multiple surrogate markers of low $N_{glomer}$ including low birth weight, prematurity, short stature, small kidneys, gestational diabetes and evidence of large glomeruli [5], in order to factor in the assessment of CKD risk. However, there is little that can currently be offered to these patients in order to prevent disease, but the combination of low $N_{glomer}$ and large body size has some potential, which is supported by strategies to reduce weight in severely obese patients with obesity-related glomerulopathy [60-62]. We postulate that body size modulation (i.e. weight control) may be particularly effective in subjects at risk of having low $N_{glomer}$ (i.e. low birth weight) [4, 5, 8, 27]. This hypothesis is supported by a recent publication from Silverwood et al. [63-65], who provided clinical evidence from a large British cohort that the development of overweight in those born with low birth weight is closely associated with reductions in renal function in adult life. In our study cohort, BMI and BSA were both independent predictors of glomerular hypertrophy in a setting of low $N_{glomer}$ providing a more general approach to body size modulation. It is noteworthy that body size can reflect fat and/or muscle mass, both of which have a direct effect on renal function [66]. Consequently, our findings may not be limited to a setting of obesity and could also be associated with increases in muscle mass (i.e. professional athletes). However, our findings illustrate that while the combination of low $N_{glomer}$ and large body size is powerful, race also plays a very important role in glomerular hypertrophy.

Zimanyi et al. [29] suggested that African American males were ‘not protected’ from glomerular hypertrophy by high $N_{glomer}$. A similar degree of glomerular hypertrophy was observed in males with a combination of low $N_{glomer}$ and high BSA in both races, suggesting that there was comparable hypertrophy. Nevertheless, multiple African Americans showed excessive glomerular hypertrophy, even in cases where no apparent reason for compensatory hypertrophy was evident. Altogether, our analysis indicates that there is anoverall excess of glomerular hypertrophy rather than lack of protection by high $N_{glomer}$. Previous reports have suggested that this excessive hypertrophy may be explained by unidentified genetic factors in African Americans [4, 29, 33, 37]. Apo-Lipo-protein 1 (Apoli) risk alleles [67] have emerged as important risk factors for the development of FSGS and HIV-associated nephropathy in African Americans [68]. We are currently assessing associations between ApolI risk alleles, nephron number and glomerular volume in our autopsy series.

Subjects included in this study come from the largest and most comprehensive kidney autopsy series in the world. However, we acknowledge several limitations, especially the inherent limitation of a cross-sectional study design. Firstly, our autopsy data collection did not include other parameters to further analyse body dimensions (i.e. muscle mass and waist circumference), limiting our ability to define whether body size modulation reflects changes in fat or muscle mass. Secondly, Brenner et al. [9, 43] and Keller et al. [69] proposed that low $N_{glomer}$ was closely associated with the development of hypertension. While the present study shows higher rates of hypertension in subjects with low $N_{glomer}$ the study was not powered to assess the definite role of this variable. A regression analysis indicated that in these 66 subjects, the contributions of hypertension and prescribed anti-hypertensive medications to glomerular size were not as powerful as BSA and $N_{glomer}$. Finally, our focus on histological features, such as $N_{glomer}$ and IVG, could oversimplify our understanding of complex processes involving multiple factors.

In conclusion, this study demonstrates for the first time that low $N_{glomer}$ is not associated with glomerular hypertrophy in female Africans. It also shows that compensatory glomerular hypertrophy in males with low $N_{glomer}$ was closely associated with high BSA. Furthermore, glomerular size in African Americans may be confounded by multiple additional factors, possibly genetic variants associated with increased risk of CKD. The development of new non-invasive technologies to study glomerular number and size will allow longitudinal studies and evaluation in large clinical cohorts of these variables.

ACKNOWLEDGEMENTS

Part of this work was presented in poster format during the American Society of Nephrology Kidney Week, San Diego, 2012. The authors acknowledge the facilities, scientific and technical assistance from Monash Micro Imaging staff members, members of the Histology Platform at Monash University, and Susan Mott from the University of Queensland. V.G.P. received a Monash Research Graduate School Scholarship and a Faculty of Medicine International Postgraduate Scholarship to support his PhD candidature. This research was funded by grants from the National Institutes of Health (NIH R01 DK063970-01), NIH Center of Excellence in Minority Health (5P20MD000534-02), the National Medical Research Council of Australia, Janssen Cilag Australia Pty Ltd and the American Heart Association (Southeastern Affiliate).

CONFLICT OF INTEREST STATEMENT

None declared.
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Received for publication: 12.12.2013; Accepted in revised form: 11.3.2014
Chapter 5

MRI-based glomerular morphology and pathology in whole human kidneys

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
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<tr>
<td>Data collection, analysis, writing and editing of multiple drafts</td>
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The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

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<tr>
<td>Beeman, SC</td>
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<td>Cullen-McEwen, LA</td>
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<td>Charlton, JR</td>
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<td>Bertram, JF</td>
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<td>Bennett, KM</td>
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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate’s and co-authors’ contributions to this work.

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MRI-based glomerular morphology and pathology in whole human kidneys

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Submitted 11 February 2014; accepted in final form 18 March 2014

Chronic kidney disease (CKD) affects 1 in 10 American adults (10) and its prevalence is rapidly increasing. In the United States alone, ~90,000 patients will die from end-stage renal disease (ESRD) each year, and over $40 billion is spent on treatment of CKD and ESRD (23). While most of the burden of CKD is focused on ESRD, studies have shown that even the mild stages of CKD are not benign and result in both higher risks of cardiovascular disease (17) and increased healthcare costs (28). It is therefore critical to develop diagnostic tools that detect and monitor kidney disease at early stages.
Innovative Methodology

F2 MRI-BASED GLOMERULAR MORPHOLOGY AND PATHOLOGY

In vivo, also enables novel measurements of glomerular size distributions and pathologies in the kidney (3, 18). Because it is naturally occurring in mammalian tissue, CF may be relatively nontoxic (1).

Here, we present an ex vivo proof-of-concept that CF can be used as an MRI contrast agent to visualize, count, and measure the size of glomeruli in whole excised human kidneys. We further demonstrate MRI-detectable changes in glomerular and vascular morphology with renal vascular disease and hypertension.

MATERIALS AND METHODS

Sample Preparation

Cationized horse spleen ferritin (CF; molecular weight = 475 kDa) was synthesized according to Danon et al. (16). Four human kidneys were obtained at autopsy through a donor network (The International Institute for the Advancement of Medicine, Edison, NJ) after Institutional Review Board approval and informed consent. The kidneys were deemed unsuitable for transplant by the Organ Procurement Officer. A request and informed consent for research were obtained only after the kidneys were deemed unsuitable for transplant. The kidneys were flushed at autopsy with heparinized saline and stored in University of Wisconsin preservation solution and, within 24 h, the renal artery was catheterized and the kidneys were perfused with 120 ml of PBS. Three kidneys (hereafter referred to as kidneys CF1, CF2, and CF3) were perfused with 300 mg of CF in PBS per kg by kidney weight (Table 1). The kidneys were then perfused with 120 ml of PBS to remove any unbound CF and then perfused with 10% neutral buffered formalin. One kidney received no CF (as a naive control), but received the same number of PBS and formalin perfusions. A minimum of three biopsies (~1 mm3) was taken from random locations in the cortex from each kidney and prepared for immunofluorescence (IF) and transmission electron microscopy (detailed below). Targeted biopsies were also taken from the cortex of the CF2 kidney. The reasons for this are discussed below. All kidneys were stored in 10% neutral buffered formalin at 4°C.

Before imaging, the CF-labeled and unlabelled control kidneys were removed from formalin and washed three times in 500 ml PBS (for a total of 1.5 l of PBS) over 24 h. The kidneys were first imaged using a Siemens 3T Skyra MRI scanner and a 32-channel transmit/receive radio frequency head coil (Siemens, Erlangen, Germany). Whole kidneys were imaged in PBS in a sealed plastic container. A T1*-weighted (TE/TR = 20/32 ms) 3D gradient recalled echo (GRE) sequence was used to image the kidney. MR images were acquired with a resolution of 270 × 270 × 512 µm3 (field of view = 12 × 4.9 × 7.8 cm, matrix size = 448 × 182 × 144, scan time = 4.2 h in the CF-labeled kidney; FOV = 12 × 5.4 × 6.9 cm, matrix size = 448 × 200 × 128, scan time = 4 h in the unlabeled control kidney). A T2*-weighted (TE/TR = 83/2,000 ms, FA = 150, matrix size = 320 × 120 × 51, spatial resolution = 400 × 400 × 130 µm3, 24 averages, total scan time = 41.6 min) half fourier acquisition single shot turbo spin echo (HASTE) pulse sequence was also used to acquire an image of the entire control kidney to distinguish cortex and medulla. The CF-labeled kidneys did not require HASTE imaging to distinguish cortex from medulla. One slice was selected from the 3D reconstructed image data and corrected for inhomogeneities by subtracting a low-pass filtered version of the image (50 × 50 Gaussian kernel with SD = 20 voxels). The slice image intensities were normalized to a range of 0–1 and the cortex and medulla were manually segmented (directly from the T2*-weighted GRE for the CF-labeled kidney and from the coregistered HASTE T2-weighted image for the naive control). The T2*-weighted HASTE image was linearly registered to the gradient echo image using FSL FLIRT (21).

Transmission Electron Microscopy

Approximately 1 mm3 pieces of tissue were collected from the cortex of each kidney after perfusion of formalin and immediately placed in 2% glutaraldehyde/0.1 M cacodylate solution for overnight fixation. Samples were then dehydrated in graded ethanol solutions ranging from 70 to 100% and then infiltrated with and embedded in epoxy resin. The resulting blocks were cut into 70-nm sections and stained with 0.2% osmium tetroxide. Osmium tetroxide precipitates were digested with 1% periodic acid for 12 min. A Philips CM12 transmission electron microscope was used to collect images at ×53,000 magnification with an accelerating voltage of 80 kV.

Table 1. Clinical data, stereological estimates, and MRI-based data

<table>
<thead>
<tr>
<th>Age, y</th>
<th>Gender</th>
<th>Race</th>
<th>Cause of Death</th>
<th>Initial Creatinine, mg/dl</th>
<th>Initial GFR, ml/min×1.73 m2</th>
<th>Peak Creatinine, mg/dl</th>
<th>Last Creatinine, mg/dl</th>
<th>Nglomer (%)</th>
<th>Vglomer (×10−6 mm3)</th>
<th>Nglomer %</th>
<th>Vglomer %</th>
</tr>
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<tbody>
<tr>
<td>CF1 68</td>
<td>M</td>
<td>C</td>
<td>Cardiac arrest</td>
<td>1.6</td>
<td>43</td>
<td>2.9</td>
<td>2.5</td>
<td>1.13</td>
<td>5.01</td>
<td>167</td>
<td>1.27</td>
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<tr>
<td>CF2 45</td>
<td>F</td>
<td>AA</td>
<td>Hypertensive stroke</td>
<td>1.1</td>
<td>65</td>
<td>2.7</td>
<td>2.7</td>
<td>0.74</td>
<td>4.68</td>
<td>110</td>
<td>0.92</td>
</tr>
<tr>
<td>CF3 37</td>
<td>F</td>
<td>C</td>
<td>Cardiac arrest</td>
<td>1.9</td>
<td>30</td>
<td>6.05</td>
<td>6.05</td>
<td>1.46</td>
<td>2.82</td>
<td>186</td>
<td>1.52</td>
</tr>
<tr>
<td>Crl 55</td>
<td>F</td>
<td>C</td>
<td>Stroke</td>
<td>1.6</td>
<td>45</td>
<td>1.8</td>
<td>1.2</td>
<td>—</td>
<td>—</td>
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Stereological estimates and magnetic resonance imaging (MRI)-based measurements for total nephron number (Nglomer) and mean glomerular volume (Vglomer) follow a similar trend, although differences between the 2 techniques are apparent. It is important to note that, due to the difference in the number of glomeruli sampled using each method and the heterogeneous nature of human kidneys, we do not expect these 2 measurements to be in perfect agreement. *MRDRI Study Equation was used to calculate glomerular filtration rate (GFR).
for 4 h and stored in PBS overnight. The samples were cryoprotected in 15% sucrose followed by 30% sucrose and then rapidly frozen to −80°C and cut into 35-μm sections. The sections were washed in PBS, permeabilized with 0.5% Triton X-100 (Sigma, St. Louis, MO), incubated in rabbit anti-horse spleen ferritin (Sigma), immunostained with an Alexa 594 goat anti-rabbit secondary antibody and 4′,6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA), and imaged on a Zeiss 710 laser-scanning confocal microscope.

Formalin-fixed tissue. A second round of IF was performed in formalin-fixed tissue after MRI and stereological analysis. Two approaches were used for tissue sampling and paraffin embedding: 1) targeted sampling and 2) random sampling. Targeted sampling, based on MRI, was used for analysis of the CF2 kidney to extract 2-mm³ samples from areas of cortex with good ferritin labeling and areas of cortex with poor ferritin labeling. For CF1 and CF3, similar size blocks were randomly cut from the cortex of the formalin-fixed tissue. All formalin-fixed tissue samples were embedded in paraffin. Three serial sections (4-μm thick each) were cut from each block. The first section was used for IF while the second section was used for periodic acid Schiff (PAS) staining. For IF, following sectioning, sections were rehydrated in 100% ethanol (5 min), 70% ethanol (5 min), and then PBS (5 min). Sections were then subjected to an antigen retrieval step, which involved immersion in Target Retrieval Solution (DAKO, S1699) for 20 min at a controlled temperature of 90°C in a DAKO PT Link PT10126 system. After being cooled, slides were washed in buffer (DAKO, K3007) and then in 1% filtered BSA in PBS for 1 h. Sections were then immunostained using an antibody against Wilms’ Tumor-1 (WT1) antigen (monoclonal mouse anti-human WT1-DAKO, M356101, clone 6F-H2), a well-known podocyte marker, that allowed us to confirm glomerular localization and the same rabbit anti-horse spleen ferritin (Sigma), as previously described. After 1-h incubation at room temperature, sections were labeled with goat anti-mouse Alexa 488 (1:2,000; Invitrogen A-11008) and goat anti-rabbit Alexa 555 (1:1,000; Invitrogen A-11001) for another hour at room temperature under light protection. Finally, Prolong Gold with DAPI (Invitrogen P-36931; anti-fade mounting medium) was used for permanent coverslipping and left for 24 h. Confocal images were taken on a Leica SP5 laser confocal microscope (Leica Microsystems, Mannheim, Germany). Images were obtained using a ×40 objective lens (1.25 NA), using sequential imaging for 488 nm, 555 nm and UV light.

Histopathology and Stereology

The MRI-based measurements of \( N_{\text{glomerulus}} \) and \( V_{\text{glomerulus}} \) were validated (after MRI) using the physical dissector/fractionator design-based stereological method described by Cullen-McEwen et al. (12–14). In brief, kidneys were weighed and a series of sampling and subsampling steps were applied to select a systematic uniform random sample of 10–15 tissue blocks from the cortex. These blocks were embedded in glycolmethacrylate (Kulzer GmbH), serially sectioned at 20 μm, and every 10th and 11th section pair was collected and stained with PAS. The section pairs were viewed with a pair of light microscopes modified for projection. Glomeruli present in one section (the reference section) but not in the paired section (look-up section) were counted according to the dissector principle, stereological grid points overlying glomeruli were counted and used to estimate \( V_{\text{glomerulus}} \).

PAS-stained glycolmethacrylate sections were assessed by a specialist renal pathologist (JD). Sections from ~10 blocks per kidney were examined. Seventy two, 61, and 66 glomeruli were assessed in kidneys CF1, CF2, and CF3, respectively.

Image Processing

\( N_{\text{glomerulus}} \) and the individual volumes of all glomeruli were calculated from the MR images using an in-house algorithm written for MATLAB (The Mathworks, Natick, MA). First, a Hessian for each voxel of the raw MRI volume (in 3D) was used to flag candidate glomerular regions and discern glomeruli in close proximity to each other. This step populated candidate regions and, as a result, dramatically reduced the data size. Five features, including edge intensity, edge persistence, region volumes, shape index, and the Laplacian of Gaussian, were extracted to remove false positive glomeruli. With those features, a Gaussian mixture model clustering algorithm was used to group candidate regions (black dots) into several clusters throughout the volume. Next, all clusters were overlain individually onto the original MRI volume, and clusters that did not identify populations of glomeruli were identified manually and eliminated from further analyses. The remaining black dots were counted as glomeruli and their sizes were measured based on the number of voxels comprising each dot. Clusters of black dots in the control kidney of similar locations and appearance to those of CF-labeled kidneys were counted as false glomeruli to quantify the negative contribution of blood artifact.

To validate the automated algorithm, we counted, by eye, the number of labeled glomeruli in six 35-μm³ sections of the original magnitude MRI volume. The same 35-μm³ sections were then compared with the glomeruli in the same images identified by the algorithm. These glomerular counts were compared using a paired Student’s two-tailed t-test and were well correlated (\( R^2 = 0.88 \)).

Image Texture Analysis

We performed image texture analysis to assess the pattern of glomerular labeling in the MR images of CF-labeled kidneys. We manually drew 16 lines (each 64 voxels in length) through the cortex of each kidney in 7T MRI volumes (all of which have the same FOV and matrix size) in ImageJ and plotted the signal line profile. The location of each line profile was randomly chosen and each profile was oriented through glomeruli that appeared to originate from the same interlobular artery. The spatial power spectrum from each line profile was calculated based on the Fast Fourier Transform in MATLAB. Spatial power spectra of CF-perfused and control kidneys were compared at each spatial frequency using Student’s two-tailed t-tests (\( p < 0.05 \)).

Statistics

Statistical analyses were calculated in MATLAB as either two-sample or paired two-tailed Student’s t-tests to test the hypothesis that the mean difference between groups is zero (\( \alpha = 0.05 \)).

RESULTS

To investigate the use of CF as a glomerulus-specific MRI contrast agent in humans, CF was injected into the renal artery of three viable (but untransplantable) human donor kidneys within 24 h of resection. Saline was injected into one kidney instead of CF as a control. The CF-labeled kidneys are hereafter referred to as kidney CF1, CF2, and CF3. Donor data were investigated to establish possible reasons for any variability in nephron number and CF accumulation in the kidneys measured by MRI (see Pathology section). These data are shown in Table 1. Notably, the donor of kidney CF2 suffered from severe, untreated hypertension, and the donor of kidney CF1 suffered from mild, treated hypertension.

MRI

We imaged the intact, fixed donor kidneys on a 7T MRI scanner using a 3D GRE pulse sequence. As shown in Fig. 1A, the MR images exhibited dark spots throughout the renal cortex of the CF-labeled kidneys. Each dark spot in the cortex is ~50–80% darker than the surrounding cortex. These dark
Fig. 1. Intravenously injected cationized ferritin (CF) specifically labels glomeruli in perfused human donor kidneys, making them visible with 7T magnetic resonance imaging (MRI). MRI of CF-labeled human kidneys reveals punctate dark spots throughout the cortex (A). A naive control kidney showed no such spots but did show minimal signal loss caused by residual blood (D). Immunofluorescence confirmed the accumulation of CF (red) in glomeruli (B, top arrow) and leakage of CF into tubules of CF-perfused kidneys (B, bottom arrow). Naive control glomeruli remained clear of CF-related immunofluorescence (E). Transmission electron microscopy (TEM) confirmed the accumulation of CF in the glomerular basement membrane (GBM) and endothelial glycoscalyx (arrows, C). The glomerular capillary walls of the naive control kidney were clear of any punctate TEM signal darkening associated with the accumulation of CF (F). White scale bars = 50 µm. Black scale bars = 0.2 µm.

The labeled glomeruli defined the boundary between the cortex and medulla and revealed the individual lobes and papillas of the kidney. The specific binding of CF to the glomerulus was confirmed with IF microscopy (Fig. 1, B, E). Transmission electron microscopy showed CF bound to the GBM as well as to the glycoscalyx of glomerular endothelial cells (Fig. 1C). Transmission electron microscopy did not reveal this staining in control kidneys (Fig. 1F).

To assess the possibility of detecting glomeruli in typical clinical MRI systems, CF-labeled kidneys were also scanned at lower resolution on a clinical 3T MRI scanner (Fig. 2). While individual glomeruli were not visible at this lower resolution, the average image magnitude in the cortex of CF-labeled kidneys was ~20% lower than in the medulla. Minimal difference between image magnitude in the cortex and medulla (~2%) was seen in the unlabeled control kidney. Thus, CF labeling can be detected with typical clinical MRI systems by measuring the ratio of cortical to medullary image intensity.

Leakage of CF through the GBM into the proximal tubule was viable in MRI and IF images of kidneys CF1 and CF2 (Fig. 3A, Fig. 4G, Fig. 5A and D, and zoomed MRI panels of Fig. 6). The leakage of CF past the glomerular capillary wall appeared as diffuse CF accumulation and MRI signal darkening, similar to leakage previously observed in a rat model of focal and segmental glomerulosclerosis (5). CF was visible by IF in the tubules (Fig. 1B, lower arrow) and Bowman’s capsules (Fig. 4V) of kidney CF1, consistent with CF leakage in the accumulation of CF (red) in glomeruli (Fig. 1C) and leakage of CF into tubules of CF-perfused kidneys (Fig. 1B).

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kidneys CF1 and CF2. Based on MRI, large regions of the cortex of kidney CF2 lacked CF-labeled glomeruli (Fig. 3A) and IF (Fig. 4, E–H). Histopathological examination of this kidney suggested that this was due to severe glomerular and arteriolar sclerosis that prevented perfusion of those areas of the cortex (Fig. 3). In comparison, the MR image darkening in glomeruli of kidney CF3 (histopathologically deemed the healthiest of the group) was punctate and appeared throughout the entire cortex of the kidney (see Fig. 5G), suggesting minimal protein leakage and vascular damage. This was supported by IF (Fig. 4, A–D).

IF

IF analysis with a double labeling for ferritin and WT-1 revealed that the CF1 kidney exhibited a large number of apparently healthy glomeruli, with moderate leakage of CF into Bowman’s capsule. The CF2 kidney contained a large number of poorly labeled glomeruli (Fig. 4, E–H). CF2 also contained a large number of glomeruli with an expanded Bowman’s space, collapsed glomerular tufts, and WT-1-positive cells—all features compatible with atubular glomeruli or obstructed collecting ducts (Fig. 4, I–L). There was heavy leakage of CF into Bowman’s space and signs of vascular remodeling or thickening of the vessel wall (Fig. 4N). Signs of vascular remodeling (Fig. 4P) were particularly apparent in the regions of the CF2 kidney that lacked CF-related MRI signal changes. Interestingly, remodeling vessels appeared to accumulate a large amount of CF in this kidney, suggesting highly charged endothelial surfaces. The CF3 kidney was dominated by healthy glomeruli, with a large accumulation of CF in the GBM and light accumulation of CF in the peritubular capillaries and Bowman’s capsules (Fig. 4, A–D).

Histopathology

Histopathology of kidney CF1 revealed four sclerotic glomeruli of the 72 examined. There was widespread patchy fibrosis and tubular dilation and atrophy with mild diffuse lymphohistiocytic infiltration within the interstitium. The arteries were sclerotic with variable hyalinosis. Overall, there was minimal nephrosclerosis and acute tubular injury.

Five totally sclerosed glomeruli of the 61 were examined and one with perihilar segmental sclerosis in kidney CF2. In the regions of CF-labeled tissue (Fig. 3B), the interstitium had widespread mild to patchy fibrosis, tubules were mildly dilated and atrophic, and a moderate lymphohistiocytic infiltrate was present that included eosinophils. The arterioles were tortuous and showed marked hyalinosis with intimal sclerosis. Within the unlabeled regions of CF2, the degree and number of severely sclerotic glomeruli were striking (Fig. 3C). Vascular involvement was evident, with both the arteries and arterioles severely thickened. In some unlabeled areas, it was impossible to distinguish sclerotic glomeruli from obstructed arterioles. The tubules in the unlabeled areas were unaffected overall, but did occasionally contain cast material. We concluded that the observed lack of CF-labeled glomeruli in some regions of kidney CF2 was correlated with focal sclerosis and vascular damage at those locations.

Kidney CF3 had one sclerotic glomerulus of the 66 glomeruli examined, with no mesangial proliferation or segmental sclerosis within the glomeruli. The interstitium showed slight fibrosis and tubular atrophy with minimal lymphohistiocytic interstitial inflammatory infiltrate. The arteries were either normal or had mild sclerosis of the intima. The tubules were mildly dilated with scattered uromodulin casts. There were only very mild changes of acute tubular injury and very mild background nephro sclerosis.

Quantitative Morphology

We developed and applied custom software to measure glomerular number and individual glomerular volume from the MR images. The custom 3D image processing software identified (Fig. 5, B, E, H, K) and measured the size of labeled glomeruli (Fig. 5, C, F, I, L) in the MRI volumes of CF-labeled kidneys.

The number of glomeruli identified in the MR images by the software yielded the total apparent number of glomeruli per kidney (\( N_{\text{glom}} \)). These data were compared with stereological estimates of \( N_{\text{glom}} \) (Table 1). Both MRI- and stereology-based measurements were consistent with the range of \( N_{\text{glom}} \) reported in the literature (24). The algorithm counted 0.057 \( \times 10^6 \) false positives.
glomeruli in the one naive control kidney, yielding a false positive rate of the image processing program of 6%.

Using the same software, we estimated the median apparent glomerular volumes ($\tilde{v}_{glom}$) using the MR images and compared them with stereological estimates (Table 1). These median volumes are consistent with those reported in the literature (24). The MRI-based measurements were used to generate the glomerular size distribution for each CF-labeled kidney, which cannot be obtained with other techniques (Fig. 5, C, F, I, L). We observed a large number of glomeruli in these distributions with volumes of $2.4 \times 10^{-3}$ mm$^3$ or less. This was unexpected, because prior stereological estimates of glomerular volumes in human kidneys suggest that only ~10% of the total number of glomeruli in a kidney should have volumes this small (data not shown). In MRI, these glomeruli represented ~30% of the total number of glomeruli.

Image Texture Analysis

To detect morphological differences between MRI volumes of CF-labeled donor kidneys (Fig. 6), we performed image texture analysis. This analysis included spatial power spectra.
associated with line profiles randomly drawn in the cortex in the MR images. The line signal profiles in the CF1 kidney, which showed only mild nephrosclerosis, were composed of a mix of high- and low-frequency oscillations with CF-related spatial spectral peaks at \( k = 0.8 \text{ mm}^{-1} \) (4.5% of total signal power) and \( k = 1.2 \text{ mm}^{-1} \) (5% of total signal power). The line signal profiles in kidney CF2, histopathologically assessed as the least healthy kidney of the group, were composed of low-frequency CF-related oscillations corresponding to a CF-related spatial spectral peak at \( k = 0.8 \text{ mm}^{-1} \), which accounted for 5% of the total signal power along the line profiles. The line signal profiles in CF3, defined by histopathology as the healthiest kidney of the group, demonstrated high-spatial frequency oscillations between \( k = 1.2 \) and \( 1.5 \text{ mm}^{-1} \) and account for 10% of the total signal power along the line profiles.

**DISCUSSION**

This work demonstrates that individual glomeruli in human kidneys can be detected using intravenous injection of CF, followed by MRI. With this approach, the apparent number \( (aN_{\text{glom}}) \) and volume \( (aV_{\text{glom}}) \) of glomeruli can be measured in the whole kidney. We refer to these as "apparent" measurements of glomerular number and volume because they are based on indirect detection of glomeruli by an exogenous agent and on a computer algorithm to measure the sites of agent accumulation. Nevertheless, this technique expands the number of glomeruli that can be practically sampled by many orders of magnitude; from hundreds of glomeruli using stereology to all functioning glomeruli in the kidney (of the order of \( 10^5-10^6 \) glomeruli). The MRI technique has potential for direct translation to clinical practice to aid in the evaluation of transplant allografts, the diagnosis of kidney disease, and the quantitation of nephron endowment in children born early or with low birth weight.

MRI-based measurements of \( aN_{\text{glom}} \) and \( aV_{\text{glom}} \) for CF1 and CF3 agreed well with the estimates obtained using stereology. We note that, due to the difference in the number of glomeruli sampled using each method and the heterogenous nature of human kidneys, we do not expect these two measurements to be in perfect agreement. Furthermore, we counted 57,000 glomerulus-like dark spots in the control kidney, most of which were likely attributed to residual blood. This false positive rate of this counting technique is thus of the order of previous reports in rat kidneys (3).

Fig. 5. Glomeruli are made visible in all 3 CF-labeled kidneys (A, D, G) and the glomerular segmentation algorithm was able to identify, count, and measure the size of labeled glomeruli (B, E, H). Identified glomeruli are assigned an arbitrary color for visualization purposes in these panels. The majority of regions defined as glomeruli by the algorithm exist in the cortical and juxtamedullary regions of the representative slice. The control kidney (J, K) shows very few regions defined as "glomeruli"—most of which are likely attributed to residual blood. The MRI-measured apparent glomerular volume \( (aV_{\text{glom}}) \) distribution for each kidney is shown, along with a gray line showing the median MRI-measured \( aV_{\text{glom}} \) and a black line showing the \( V_{\text{glom}} \) estimate obtained using stereology (C, F, I, and L). Note that no stereological measurement is available for the control kidney.
Fig. 6. Line profiles (16 per kidney) were drawn through the cortex of each CF-labeled kidney in 7T MRI volumes (all of which have the same FOV and matrix size). Here, the line signal profiles of the solid white profiles are plotted (A, C, and E) and the mean power spectrum for the 16 line profiles is shown (B, D, and F). Black traces are data from CF-labeled kidneys and gray traces are data from the naive control. Arrows point to spatial spectral peaks of particular interest. Qualitatively, the line profiles for the CF-labeled kidneys appear different from one another, with the CF3 line profiles being mostly composed of relatively high-frequency oscillations, the CF2 oscillations being mostly composed of relatively low-frequency oscillations, and the CF1 kidney being composed of a mixture of high- and low-frequency components. The line profile signal changes associated with the appearance and disappearance of sites of CF accumulation in the CF1 kidney correspond to 2 spectral peaks, one at \( k = 0.8 \text{ mm}^{-1} \) (4.5% of total signal) and one at \( k = 1.2 \text{ mm}^{-1} \) (5% of total signal). B. The line profile signal changes associated with CF accumulation in the CF2 kidney correspond to only a low-frequency spatial signal oscillation at \( k = 0.8 \text{ mm}^{-1} \) and account for 5% of the total signal along the line profiles (D). The line profile signal changes associated with the accumulation of CF in the CF3 kidney correspond only to high-frequency spatial oscillations between \( k = 3.2 \text{ and } 3.5 \text{ mm}^{-1} \), which account for 10% of the total signal along the line profiles (F). Histopathology showed that CF2 had substantial nephrosclerosis, CF1 had mild nephrosclerosis, and CF3 had minimal nephrosclerosis; therefore, the prominence of low-frequency spectral peaks may suggest the advancement of nephrosclerosis. Stars represent a statistically significant difference between the CF-labeled kidney and the naive control (\( P < 0.04 \)) and arrows denote peaks of particular interest. Error bars represent means ± 1 SD between power spectra of 16 randomly chosen line profiles.
CF in nonglomerular structures or partial CF labeling in some glomeruli, or both. Future work will focus on improving image analysis and MRI acquisition to reduce the number of falsely identified glomeruli. It may be possible to exclude these erroneous glomeruli by setting a strict lower image processing threshold on the IGV measurements. Our data illustrate that caution is required in adjusting this threshold in the future to ensure that IGV is not simply adjusted to give a “correct” result. Nonetheless, the intrarebral distribution of glomerular volume could be used as a powerful new parameter for assessing glomerular hypertrophy and shrinkage in health and disease.

We quantified the spatial distribution of CF accumulation in the kidney by image texture analysis. The CF-related image signal varies with the kidney thickness and the amount of accumulated CF, the least reported nephrosclerosis—was punctuate and was associated with spatial power spectral peaks at k = 1.2 and 1.5 mm⁻¹. With profound arteriolar sclerosis and nephrosclerosis, as in kidney CF2, the CF-related signal darkening appeared diffuse and was associated with a spatial power spectral peak at k = 0.8 mm⁻¹. Previous work in rats showed that this diffuse labeling, quantified using the spatial power spectrum, can result from leakage of protein past the glomerular capillary wall into Bowman’s space and the proximal tubule (5). This analysis is supported by the histopathological and spectral analyses of kidney CF1, which exhibited only mild nephrosclerosis and spectral peaks at both k = 0.8 and 1.2 mm⁻¹, suggesting populations of healthy (k = 0.8 mm⁻¹) and sclerotic (k = 1.2 mm⁻¹) glomeruli. Image texture analysis may prove useful to quantify morphological changes with disease progression.

CF creates contrast in T₂*-weighted MRI by dephasing the spins of water protons surrounding the site of CF accumulation. The volume over which this dephasing is seen in T₂*-weighted MRI images depends on the amount of accumulated CF and the image acquisition parameters. It is thus important to consider imaging parameters and to minimize CF dosage when measuring aVὲh,m with T₂*-weighted MRI. While this work used a large CF dose of 300 mg/kg kidney wt, intravenous doses of just 0.6–1 mg/kg body wt of CF are sufficient to visualize rat glomeruli with T₂*-weighted MRI and have minimal effects on the kidney, liver, and immune function biomarkers (1–3). Substantial work must be done in the future to optimize dosing of donor kidney with CF. Furthermore, recent advances in MRI contrast agent design have also opened the door to labeling glomeruli with a highly sensitive T₁-shortening (bright) MRI "para-CF" contrast agents (11), allowing for improved in vivo detection of glomeruli with T₁-weighted MRI and elimination of the dephasing artifacts found in T₂*-weighted MRI that might affect volume measurements, and a further 100-fold reduction in required dose. Such an agent would greatly improve glomerular detection in vivo against the dark blood background and allow for doses that may have a trivial effect on the inherent charge of the GBM.

The ability to clinically measure glomerular morphology and local protein leakage has the potential to directly improve patient care and clinical outcomes. Measurements of glomerular morphology could be used to assess the viability of kidneys from both living and deceased donors, ensuring that a donor kidney has sufficient filtration surface area. The relationship between glomerular morphology and kidney viability, measured by in vivo GFR and survival rates, will be the subject of future work in preclinical models of kidney transplants. Noninvasive glomerular morphological measurements would also allow younger recipients to receive kidneys possessing a nephron number sufficient to match their future life span. Individuals at risk for CKD could receive an individualized risk assessment using this technique, enabling early detection and regular monitoring of kidney disease. Still, the use of such a technique in the clinic requires substantial work to address scan time and contrast agent toxicity. Many of these studies are already underway. Development of highly sensitive, T₁-shortening (bright) contrast agents (11) and advancements in radio frequency hardware for high-resolution in vivo MRI of the kidney (26) have made it possible to visualize glomeruli in vivo in a matter of minutes, although specialized contrast agents and semi-invasive hardware are currently required to do so. Furthermore, initial study of the toxicity and biodistribution of CF (1) suggests MRI-detectable doses of CF to be minimally toxic. Production of recombinant human ferritin may further reduce toxicity (30). It will also be increasingly important to determine the minimum dose of CF needed to detect glomeruli in human studies with MRI. Based on our previous in vivo studies, and accounting for allometric differences between rats and humans, we approximate the in vivo detectable limit of intravenous CF in humans to be 0.56 mg/kg.

In conclusion, glomerular number and volume in viable human kidneys can be visualized and measured using MRI. To the best of our knowledge, this is the first technique to measure the volume of every glomerulus in the human kidney and to identify large regions of arteriolar and glomerular sclerosis. This technique is nondestructive and therefore has the potential for translation to the clinic. This study is thus a first step toward characterizing human kidney glomeruli in vivo.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the joint Barrow Neurological Institute–Arizona State University Center for Preclinical Imaging and the support from the Monash Biomedical Imaging Research Platform. We also acknowledge support from David Lowry at the ASU Electron Microscopy Facility, Joseph Georges at the Translational Genomics Research Institute (confocal microscopy), and Monash/Micro Imaging (confocal microscopy). Histological sectioning and staining for pathological analysis were performed by staff at the Monash Histology Platform. We acknowledge the outstanding staff of the International Institute for the Advancement of Medicine of the Musculoskeletal Tissue Foundation for tissue procurement and support.

GRANTS

G. F. Egan is a National Health and Medical Research Council Principal Research Fellow. This work was funded by a grant from the National Institutes of Health (NIH) Diabetic Complications Consortium and by NIH Grant DK-091722.

DISCLOSURES

K.M. Bennett owns Nanodiagnostics, LLC.

AUTHOR CONTRIBUTIONS


REFERENCES

REFERENCES


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Chapter 6

Design-based stereological methods for estimating numbers of glomerular podocytes

Declaration for Thesis Chapter 6

Declaration by candidate

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
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<tr>
<td>Data collection, analysis, and writing and editing of multiple drafts</td>
<td>80%</td>
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The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of contribution</th>
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<tbody>
<tr>
<td>Douglas-Denton, RN</td>
<td>Data collection</td>
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<td>Cullen-McEwen, LA</td>
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<td>McNamara, BJ</td>
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<td>Hughson, MD</td>
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<td>Hoy, WE</td>
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<td>Nyengaard, JR</td>
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<td>Bertram, JF</td>
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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate’s and co-authors’ contributions to this work.

| Candidate’s Signature | Date: 21-5-14
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| Main Supervisor’s Signature | Date: 21-5-14
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Design-based stereological methods for estimating numbers of glomerular podocytes

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\textbf{ARTICLE INFO}

\textbf{Article info:}
Received 12 December 2012
Received in revised form 27 March 2013
Accepted 15 April 2013

\textbf{Keywords:}
Podocyte
Glomerular volume
Stereoology
Immunohistochemistry and confocal microscopy

\textbf{SUMMARY}

The podocyte depletion hypothesis has emerged as a unifying concept in glomerular pathology. According to this hypothesis podocyte depletion may be absolute (decrease in number of healthy mature podocytes), relative (fewer podocytes per unit of glomerular volume) or involve alterations to the specialized podocyte architecture (such as foot process effacement). To study and understand podocyte depletion it is important to be able to accurately and precisely count these cells. Here we present new design-based stereological methods for estimating podocyte number in individual glomeruli of known volume, and in average glomeruli. Both methods involve serial histological sectioning, triple label immunohistochemistry, laser confocal microscopy and cell counting with the optical dissector/fractionator.

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1. Introduction

Podocytes are exclusively located within renal glomeruli (Fig. 1A and B) and together with glomerular endothelial cells and the glomerular basement membrane form the glomerular filtration barrier. Alterations to the integrity of podocytes and the filtration barrier are involved in the development and progression of a range of renal pathologies (Kriz and Endlich, 2012).

Podocytes have a highly specialized structure, consisting of a cell body (containing the nucleus, mitochondria, Golgi apparatus, rough endoplasmic reticulum and lysosomes) from which emanate large cytoplasmic processes (Fig. 1C). Foot processes (pediciles) in turn emanate from the large cytoplasmic processes and are attached to the basement membrane. Filtration slits (Fig. 1D) are located between adjacent foot processes and feature a slit diaphragm which regulates the flow of water and small molecules across the filtration barrier. In addition to their key roles as components of the glomerular filtration barrier, podocytes synthesize and secrete a range of growth factors as well as components of the glomerular basement membrane (Jefferson et al., 2011).

It has been shown for many years that podocyte dysfunction is associated with a large number of primary and secondary glomerular pathologies including minimal change disease, focal and segmental glomerulosclerosis (FSGS), diabetic nephropathy, collapsing glomerulopathy, diffuse mesangial sclerosis, congenital nephrotic syndrome of the Finnish type, Alport’s syndrome and obesity-related glomerulosclerosis (Wiggins, 2007). Despite the differences in etiology, glomerular pathology and clinical severity of these conditions, the podocyte depletion hypothesis has emerged in recent years as a unifying concept in glomerular pathology (Wiggins, 2007; Wharram et al., 2005; Fukuda et al., 2012; Kriz et al., 1996, 1998; Kriz, 1997, 2002). In brief, this hypothesis proposes that podocyte depletion, whether it be absolute, relative or involving phenotypic change, renders glomeruli susceptible to subsequent pathological change. Absolute podocyte depletion involves a loss of podocytes, via apoptosis, necrosis or detachment from the basement membrane (for a comprehensive review see Tharaux and Huber, 2012). Relative podocyte depletion occurs when a finite number of podocytes is required to cover an expanded glomerular filtration surface area or maintain a hypertrophied glomerular volume. Podocyte phenotypic changes include foot process effacement and cellular hypertrophy. The podocyte depletion hypothesis is predicated on the understanding that podocytes have little or no capacity for proliferation and thereby replacement. However, recent evidence suggests that some degree of podocyte

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http://dx.doi.org/10.1016/j.aanat.2013.04.007
replacement may be possible via the proliferation and differentia- 
tion of a subpopulation of parietal epithelial cells (PECs) located 
close to the urinary pole (Sagginato et al., 2006; Ronconi et al., 2009; 
Appel et al., 2009).

To study and understand podocyte depletion it is important to 
be able to accurately and precisely count these cells. Here we 
present new design-based stereological methods for estimating 
podocyte number in individual glomeruli of known volume, and in 
average glomeruli. Before describing these methods, we briefly 
discuss the methods currently available for estimating podocyte number.

2. Current methods for quantifying podocyte number

A range of methods are currently employed to estimate podocyte number. Perhaps the most commonly used method involves the counting and reporting of numbers of podocyte nuclear 
profiles (the bits and pieces of nuclei seen in histological sections) 
per glomerular cross-section ($N_{\text{pNC}}$) or per unit area of glomeru- 
lar section ($N_{\text{pLA}}$). In both cases, it is important to note that it is not 
podocytes that are being counted but rather podocyte nuclear pro-
files. While these two approaches may appear valid, it turns out 
that the number of podocyte nuclear profiles seen in a section is not 
only related to the number of podocyte nuclei (and assuming one 
nucleus per podocyte, the number of podocytes) present, but also 
to podocyte nuclear shape and size, and section thickness. More-
over, neither method provides an estimate of the total number of 
podocytes in a glomerulus.

To overcome the problems above, a range of model-based stereological methods were developed in the twentieth century (Fodor and Flex, 1944; Wicksell, 1925; Abercrombie, 1946; 
Dehoff and Rhines, 1961; Weibel and Gomez, 1962). These meth-
ods provide estimates of podocyte nuclear numerical density (and 
thereby podocyte numerical density) in glomeruli ($N_{\text{pNC}}$, number of 
podocytes per unit volume of glomerulus), when $N_{\text{pNC}}$ is multiplied 
by glomerular volume we obtain an estimate of the total number of 
podocytes in a glomerulus ($N_{\text{pGLM}}$). However, these methods are designated “model-based” because they require knowledge 
of the geometry (mean caliper diameter, size, size distribution, 
shape) of the podocyte nucleus. Generally, values for these geometric 
parameters are assumed rather than measured, and therefore 
to the extent that these geometric assumptions are incorrect, the 
resultant estimates of podocyte number are biased.

In 1984, the publication of the disector method by Sterio (1984) 
revolutionized stereology because, for the first time, knowledge of 
particle (a 3-dimensional object such as a podocyte nucleus) geom-
metry was not required to estimate number. The disector samples 
particles such as podocyte nuclei with equal opportunity, regard-
less of their size, size distribution, orientation or shape. The first 
iteration of the disector required the comparison of features in two 
physical sections— the physical disector. This method has been used 
by several groups to successfully count podocytes in rat (Bai and 
Bosgen, 2011; Bertram et al., 1992) and human glomeruli (White 
et al., 2002; White and Bilus, 2004) at both the light and elec-
tron microscopic levels. In 1986, Gundersen described the optical 
disector, with which optical sections through tissue are compared 
and particles of interest then counted using the disector principle.
These optical sections can be obtained with confocal microscopy (Peterson, 1999) or using bright field microscopes fitted with high numerical aperture condenser and objective lenses (Bertram and Mur compass, 1992).

A critical requirement for any method used to count podocytes is the ability to unambiguously identify podocytes. Podocyte identification can be achieved by a number of means including serial sectioning (Ba and Bangs, 2011), electron microscopy (Bertram et al., 1997; White et al., 2001), and the use of specific antibodies (Wharram et al., 2005; Sanden et al., 2003). It is important to note that when counting podocytes with the methods mentioned above, including optical dissectors, it is podocyte nuclei that are actually counted. In this regard, it is worth noting that binucleated podocytes have been described in several pathological settings (Becquemont et al., 1994; Hugo et al., 1998; Mundel et al., 1997; Richardson et al., 1998; Meyer-Schweiseger et al., 2012).

Given that podocyte depletion can either be absolute or relative, it is also desirable for any podocyte counting method to provide the volume of the glomerulus being assessed. Here we present a new design-based stereological method for estimating total podocyte number in individual (single) glomeruli of known volume, and in average glomeruli. Both methods utilize triple immunostaining for unambiguous podocyte identification, confocal imaging and cell counting with optical dissectors used in a fractonometer design. The former method also requires estimation of glomerular volume using the Cavalieri estimator (Gundersen and Jensen, 1987). The methods are described in detail below and preliminary data for human glomeruli presented.

3. A design-based method for estimating total podocyte number in individual glomeruli of known volume

3.1. Step 1. Tissue collection and preparation

Kidneys were obtained at autopsies performed at the University of Mississippi Medical Center, Jackson, MS, USA. Ethical approval was obtained in advance from the Institutional Review Board of the University of Mississippi Medical Center and Monash University Human Research Ethics Committee. Kidneys were perfusion-fixed with 10% buffered formalin, bisected and then immersed in 10% formalin. After 10 days, both halves of the kidney were cut into 4 mm slices and subsequently sampled for stereology (i.e. nephron number) processed for embedding in paraffin as previously described (Hughson et al., 2008). Fifty serial sections (each 14 μm thick; for a total of 700 μm) were obtained from each kidney (one random paraffin block).

3.2. Step 2. Glomerular sampling and volume estimation

Imaging was carried out using an Olympus DotSlide system equipped with a 20× objective (Olympus, Tokyo, Japan) and a monochrome slide Peltier cooled digital camera (Olympus Soft Imaging, Tokyo, Japan). Thirty glomeruli (10 from each of the outer, middle and inner cortex) per specimen were then sampled using physical dissectors as previously described (Puelles et al., 2012; Samuel et al., 2005). All profiles of these 30 glomeruli were imaged (until there were no more sections through that glomerulus), generally providing between 10 and 16 profiles per glomerulus. Glomerular profiles were labeled with a flag and a unique identifier (numerical labels from 1 to 30 –1–10 superficial, 11–20 middle and 21–30 juxtarrenal glomeruli). These virtual images served as maps to find all profiles of each of the 30 glomeruli during confocal microscopy. The volumes of all 30 sampled individual glomeruli per kidney were estimated using the Cavalieri estimator (Gundersen and Jensen, 1987), using:

\[
\text{Individual glomerular volume (IVG)} = \frac{1}{\text{SSF}} \cdot \frac{\text{BA}}{P} \cdot A(p) \quad (1)
\]

where SSF represents the section sampling fraction (in this case 1 because every section was measured), BA was block advance on the microtome (approximately 14 μm) and was measured as described by Dorp-Petersen et al., 2001. \(\sum P\) was the number of test grid points overlaying the glomerular tuft, and \(A(p)\) was the area associated with each grid point and was calculated using:

\[
A(p) = \left(\frac{\text{Grid size(μm)}}{\text{Final magnification(μm)}}\right)^2 \quad (2)
\]

In this case we use a quadratic 2 cm point grid (20,000 μm) and the final magnification was calculated using a virtual image of a micrometer, obtained at the same magnification (20×) as the rest of the virtual images (final magnification = 640×). It is worth mentioning that in certain cases \(A(p)\) can be adjusted in order to “do more, less well” (Gundersen and Osterby, 1981), which means that for example in subjects with evidently large glomeruli, \(A(p)\) can be modified in order to obtain a \(\sum P\) of at least 100. The estimation of glomerular volume is influenced by tissue shrinkage which is considerable in paraffin sections, probably ~50% on a volume basis (Dorp-Petersen et al., 2001). It should be noted that the estimates of total glomerular cell numbers (e.g. total number of podocytes per glomerulus) obtained with this method and cascade experimental design below are not influenced by tissue shrinkage. However, estimates of cell density (number per unit volume of glomerulus) will be affected by tissue shrinkage unless steps are taken to correct for this.

3.3. Step 3. Immunohistochemistry and confocal microscopy

Every second section (for a total of 25 sections per subject) was used for immunohistochemistry. (Note: Given the importance of unambiguous podocyte identification, we recommend including both positive and negative control slides in each batch). Following sectioning, sections were rehydrated in 100% ethanol (5× min). 70% ethanol (5× min) and then phosphate buffered saline (PBS; 5× min). Sections were then subjected to an antigen retrieval step which involved immersion in 10× citrate buffer for 20 min at 90°C (which can be achieved with an automated system, for example Dako PT Link PT10126). After cooling, slides were washed in PBS and then in 1% filtered bovine serum albumin (BSA) in PBS for 1 h. Sections were then immunostained using an antibody against Wilms’ Tumor-1 (WT1) antigen (monoclonal mouse anti-human WT1–DAKO ME35101, clone 6F12; for podocyte identification) and an antibody against von Willebrand Factor (vWF), which in this case was a polyclonal rabbit anti-human vWF (Dako A008202; for endothelial cell identification). WT1 (1:50) and vWF (1:200) were diluted in 5 ml PBS and 200 μl was applied to each slide. After placing a loose coverslip on top of each section, sections were incubated with these antibodies at 4°C overnight.

The following day, each section was washed in PBS three times for 5 min. Then, goat anti-mouse Alexa Fluor 488 (1:2000 – Invitrogen A-11008) and goat anti-rabbit Alexa Fluor 555 (1:1000 – Invitrogen A-11011) were added (200 μl per section) and left for 1 h at room temperature. After this, sections were then washed in PBS three times for 5 min. After this step, 200 μl of 4,6-diamidino-2-phenylindole (DAPI; 1:10,000 – Sigma-Aldrich D9542-10M5) was added to each section and incubated for 10 min. Then sections were washed in PBS three times for 5 min. After this step, 200 μl of 4,6-diamidino-2-phenylindole (DAPI; 1:10,000) was added to each section and incubated for 10 min. Then sections were washed in PBS three times for 5 min and carefully dried. Prolong Gold (Invitrogen P36934; anti-fade mounting medium) was used for permanent coverslipping and left for 24 h.
to cure. Finally, nail polish was used to seal the border of the coverslip, and sections were stored at 4°C.

We counted podocytes in 6 glomeruli (the 3 smallest and the 3 largest – representing the 10th and 90th percentiles respectively) from the 30 sampled glomeruli per subject used for IGV estimation (Fig. 2A). The virtual images obtained with the Dotslide workstation were uploaded onto the confocal microscope computer and used as maps to locate all profiles of each glomerulus. Every immunostained section from each of the 6 sub-sampled glomeruli per subject was imaged with a Leica SP5 laser confocal microscope (Leica Microsystems, Manheim, Germany). Images were obtained using a 40x objective lens (1.25 NA), using a set zoom (1.01), using sequential imaging for 488 nm, 555 nm and UV light (Fig. 2B). With this configuration, multiple serial optical sections 1 μm apart were collected throughout the full thickness of the 14 μm (approx.) sections (note: after immunohistochemistry, it is expected to find some section swelling; with our protocol we found this to be in the order of 1–3 μm). Images were obtained with a 3 line average and stored in a 512 × 512 pixels frame.

When counting cells with optical dissectors in thick sections, and using antibodies for cell identification, it is important to have suitable antibody penetration. As seen in Fig. 2C, our staining protocol provided good antibody penetration, as demonstrated in the Z-axis profile which was obtained using LAS AF Lite software (Leica MicroSystems, Manheim, Germany). It is also important to define guard regions at both the top and bottom of each glomerular profile to account for cutting artifacts and the possible loss of nuclei through sectioning. We defined a 3 μm guard region at the top and bottom of each section based on a pilot analysis performed in 1 μm optical sections of glomerular profiles in two subjects according to Dorph-Petersen et al. (Dorph-Petersen et al., 2001). As seen in Fig. 2D, stable nuclear counts were obtained between optical sections 4 and 16, which provides at least 8 μm of each section suitable for nuclear counting.

3.4. Step 4. Counting podocytes with optical dissectors

As shown in Fig. 3, optical dissectors were used to sample, and thereby count cells in 8 μm out of the 14 μm available for each glomerular profile. Glomerular cells were sampled and counted using optical dissectors on the series of 1 μm optical images obtained through laser confocal microscopy, stacked as a virtual
Fig. 3. How to use optical disectors to count glomerular cells. All panels (A–C) show four optical sections. We start counting on Panel A (only nuclei, DAPI+ cells); optical section 1 is used first to identify those cell nuclei that are in focus (marked with *). These nuclei are not counted because they are already in focus. Consecutive sections (1 μm) apart (optical sections 2, 3 and 4) are then used to identify nuclei that come into focus – these are the nuclei which are counted. In this case, we have identified four nuclei that come into focus in optical sections 2–4 (numbers in yellow represent the moment the nucleus comes into focus – the moment when it is counted). The second step is to “turn on” the fluorescent channels to define whether these new nuclei were podocytes or NECs (see Panels B and C). In this sequence, we identified two podocytes (2 and 4) and two NECs (1 and 3).

slide and opened using an Imaris (Schneider et al., 2012) macro that allowed us to turn on and off the three existing channels (blue – nuclei, green – podocyte cytoplasm, red – endothelial cell cytoplasm) and use a counting tool. Cell nuclei were counted when they first came into focus as described below. First, we turned off the green and red channels to leave only the blue channel showing nuclei (Fig. 3, Panel A). Those nuclei in focus in the first optical section below the top guard region were not counted, because this section was an exclusion counting plane. All nuclei that came into focus in the next 8 optical sections were then counted including those that first came into focus in the final optical section of the disector counting volume. After all newly appearing nuclei had been identified, the green and red channels were turned on (Fig. 3, Panels B and C) in order to identify podocytes (green; WT1+ cells) and non-epithelial cells (vWF+ and WT1–cells). We also counted PECs which were easily identified based on their location in Bowman’s capsule.

Cell counts were exported to an Excel spreadsheet and the following formula used to estimate the total number of podocytes in the individual glomerulus (Npod,glomer) based on the fractionator principle and optical disectors (optical fractionator):

$$N_{pod,glomer} = \frac{1}{SSF} \cdot \frac{1}{(\bar{q})} \sum Q^-$$  \hspace{1cm} (3)

where SSF represents the section sampling fraction (we used every second section so SSF = 1/2), h/T is the fraction of section thickness used for cell counting (in this case 8 μm divided by the section thickness T), and $\sum Q^-$ is the total number of podocyte nuclei counted using optical disectors. Note that T is the final section thickness, not block advance as used in Eq. (1). T can vary depending on several factors, hence we recommend that it be measured in optical sections on each glomerular profile, and that a Q–weighted average value be calculated for all profiles from each glomerulus, see (Dorff-Petersen et al., 2001). Eq. (3) can easily be modified to estimate the total number of non-epithelial cells and PECs per glomerulus and the SSF can also be modified to optimize time and efficiency.

4. Parameters

As shown in Table 1, a range of absolute and relative parameters can be estimated for individual glomeruli using the techniques described above, including the total number of podocytes (Npod,glomer), and relative podocyte parameters such as the number of podocytes per unit volume of glomerulus (Npod/glomer) and the ratio of non-epithelial cells (NECs = mesangial cells and endothelial cells) to podocytes in the glomerulus. Additional parameters and their relevance are listed in Table 1.
5. Podocyte number in human glomeruli

We are currently using this method to estimate the number of podocytes, NECs and PECs in glomeruli of known volume in human autopsy tissue. Our aim is to assess the associations between indices of podocyte depletion and risk factors for chronic kidney disease, including race, age, gender, birth weight, nephron number and body size. Values for 6 glomeruli from one subject (48 year old white American man with a body surface area of 1.94 m²) from our study of body size are shown in Table 2. In this, albeit small, preliminary study, we can see that total podocyte number ranges from 483 to 813 (a 1.7-fold range) and that the number of NECs ranges from 769 to 2004 (a 2.6-fold range). IGV ranges from 1.02 to 4.14 m² = 10³, a 4.1-fold range. As a result of these variations in podocyte number and IGV, the number of podocytes per unit volume of glomerulus ranges almost 3-fold. These preliminary data demonstrate that absolute podocyte number as well as podocyte numerical density varies widely in glomeruli from the same subject.

6. How reliable are these parameters?

The use of design-based stereology is by definition highly accurate, provided the method is applied correctly. The precision of the stereological estimates obtained can be determined by calculating the coefficient of error. Although the coefficient of error is simply defined as the mean divided by the standard deviation, the amount of sampling error expressed by the difference between an estimate and the true value is unknown, thus we need to predict the precision of these estimates.

The estimation of error variance of the optical fractionator method used for number-weighted sampling of podocytes (as well as NECs and PECs) can be performed by the quadratic approximation formula (Cainverson et al., 1999; Nyengaard, 1990). The contribution to error variance caused by the noise:

\[ \text{Var}_{\text{noise}}(\sum Q_i) = \sum Q^2 \]  

where \( \sum Q^2 \) is the number of podocytes counted. The contribution to the error variance of the estimate from the set of systematic sections using systematic uniformly random sampling (SURS), where the section number is denoted i, is:

\[ \text{Var}_{\text{SURS}}(\sum Q_i) = \frac{3}{240} \left( (\sum Q_i^2 - Q_i^3) - 4 \left( \sum Q_i^2 Q_{\text{xs}} \right) + \sum Q_i^2 Q_{\text{xx}}^2 \right) \]  

The error variance of the podocyte number estimate (also NECs and PECs) is then:

\[ CE(Q_i) = \frac{\sqrt{\text{Var}_{\text{noise}}(\sum Q_i) + \text{Var}_{\text{SURS}}(\sum Q_i)}}{\sum Q_i} \]  

As seen in Table 3, coefficients of error for podocytes and NECs were relatively stable among all 6 glomeruli, ranging from 7.3 to 9.8% (podocytes) and from 5.1 to 9.5% (NECs). Estimates for PECs showed coefficients of error between 10 and 14%. Altogether, the coefficients of error revealed that our method was remarkably precise for all three cell types.

7. Strengths and limitations of the method

The method described has several advantages over existing podocyte counting methods. First and foremost is the fact that no assumptions are required for podocyte nuclear size, size uniformity or shape – this is a design-based method (high accuracy). This is also supported by the low coefficients of error (high precision) described above. Second, a specific marker (WT1) is used to identify healthy mature podocytes. The use of multiple optical sections assists further with cell identification. And finally, the approach yields data for numerous parameters, including several indices of podocyte depletion.

The major limitation of this method is the time taken to estimate these parameters for a single glomerulus. In our hands, approximately 52 h is required for an experienced person to use this technique to estimate the parameters listed in Table 1 for 6 glomeruli (less than 10 h per glomerulus); this includes 1 h for sectioning, 6 h for immunohistochemistry (we are now using a "DMO Autostainer Plus" which requires the need for manual immunostaining); 3 h for virtual imaging and analysis; 6 h for confocal imaging; and 36 h for cell counting. Of course, the technique also requires access to the hardware and software detailed above. It should also always be borne in mind that the sensitivity of an antibody used for cell identification may alter with disease, and great care should be taken to check for this.

8. Estimating podocyte number in renal biopsies

The protocol described above can be used to count podocytes in the setting of renal pathology, such as in renal biopsies. While Fig. 4A shows a profile of a healthy glomerulus from a patient with focal and segmental glomerulosclerosis, Fig. 4B illustrates a glomerulus with a sclerosed segment. However, the method above for individual glomeruli can only be applied to biopsies if sufficient tissue is available for serial sectioning at 14 μm. When this is not the case, the method below for estimating podocyte number in average glomeruli should be employed.

9. Estimating podocyte number in average glomeruli

An alternative approach to podocyte estimation in individual (single) glomeruli is to estimate the number of podocytes in an average glomerulus. Indeed, this is the more standard stereological approach (Weibel, 1979). With this approach, we obtain the average number of podocytes per glomerulus in a kidney. We do not know the number of podocytes in any single glomerulus, but we obtain an estimate for the average number of podocytes in the population of glomeruli. This method may also be more suitable.
to podocyte counting in biopsies when limited tissue is typically available.

9.1. Step 1. Tissue preparation, sectioning and immunohistochemistry

These methods are mostly the same as above for individual glomeruli. Depending on the size of the tissue sample (for example, whole kidney or biopsy), serial sections may or may not be required to estimate the volume of the reference space \( V_{ref} \). In either case, there is no need to obtain images/measurements on multiple profiles from each glomerulus because ICV is not estimated with this technique. A single section from an approximately systematic uniform random (SUR) sample of blocks is sufficient.

9.2. Step 2. Confocal microscopy

A SUR sample of glomerular profiles is imaged on each section. The imaging is as described above for individual glomeruli. A pilot study should be conducted to determine the number of glomerular profiles required to obtain a stable estimate of podocyte number.

9.3. Step 3A. Counting podocytes in average glomeruli using a cascade experimental design

Two approaches can be used to estimate podocyte number in average glomeruli. The first involves a cascade experimental design, where \( N_{pod,average} \) is estimated using:

\[
N_{pod,average} = \frac{N_{pod,glomeruli}}{V_{glomeruli}} \cdot \frac{V_{glomeruli}}{V_{ref}}
\]

where \( N_{pod,glomeruli} \) is the numerical density of podocytes in an average glomerulus, \( V_{glomeruli}/V_{ref} \) is the volume density of glomerulus in the reference space (in this case the volume of kidney or biopsy tissue available for study), and \( V_{ref} \) is the absolute volume of the reference space. Podocytes are counted using optical dissectors as described above, \( N_{pod,glomeruli} \) is calculated using:

\[
\frac{N_{pod}}{V_{glomeruli}} = \frac{C_{pod}}{AD \cdot HD}
\]

Disector height (HD) can be 8 \( \mu \text{m} \) as above. The area of dissectors (AD) is the area of glomerular profiles analyzed. \( V_{glomeruli}/V_{ref} \) can easily be estimated by:

\[
\frac{V_{glomeruli}}{V_{ref}} = \frac{N_{glomeruli}}{N_{ref}}
\]

Table 2

t the three smallest glomeruli (10th percentile) and Glom6, Glom5 and Glom4 the three largest glomeruli (90th percentile); NEC: non-epithelial cell; PEC: peritubular epithelial cell.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Glom1</th>
<th>Glom2</th>
<th>Glom3</th>
<th>Glom4</th>
<th>Glom5</th>
<th>Glom6</th>
<th>Fold-Range</th>
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<td>Individual glomerular volume ( (ICV; 10^6 \mu m^3) )</td>
<td>1.02</td>
<td>1.07</td>
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<td>4.14</td>
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<td>483</td>
<td>504</td>
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<td>813</td>
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<td>Absolute non-epithelial cell (NEC) number per glomerulus</td>
<td>760</td>
<td>982</td>
<td>901</td>
<td>1036</td>
<td>2084</td>
<td>1939</td>
<td>3.6</td>
</tr>
<tr>
<td>Absolute peritubular epithelial cell (PEC) number per glomerulus</td>
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<td>186</td>
<td>204</td>
<td>256</td>
<td>27</td>
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<tr>
<td>Podocyte number per KIV ( (10^{-4} \cdot \mu m^2) )</td>
<td>4.75</td>
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<td>PEC/podocyte ratio</td>
<td>0.20</td>
<td>0.32</td>
<td>0.31</td>
<td>0.57</td>
<td>0.46</td>
<td>0.11</td>
<td>1.9</td>
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<tr>
<td>NEC/podocyte ratio</td>
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<td>1.56</td>
<td>1.48</td>
<td>2.79</td>
<td>2.56</td>
<td>2.19</td>
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*Note that P² of this cell population was below 100 in almost all glomeruli but Glom6.

Table 3

<table>
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<th>Glom2</th>
<th>Glom3</th>
<th>Glom4</th>
<th>Glom5</th>
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<td>8.5</td>
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<td>7.9</td>
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<tr>
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<td>12.2</td>
<td>12.6</td>
<td>10.0</td>
<td>10.4</td>
<td>12.7</td>
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Fig. 4. Counting podocytes in renal biopsies. (A) Confocal microscope image of a normal glomerulus in a patient with FSGS showing podocytes (green, WT1) and endothelial cells (red, vWF). (B) A glomerulus in a patient with FSGS with a sclerosed segment (bounded by the dotted line). No WT1+ podocytes are present in the sclerosed segment.
be estimated using the Delesse Principle (see Weibel, 1979) using:

$$P_{grom} = \frac{V_{grom}}{V_{ref}}$$

(9)

where $P_{grom}$ is the number of stereological grid points overlying glomeruli and $P_{ref}$ is the number of grid points overlying the reference tissue. Finally, $V_{ref}$ is the volume of reference tissue used and can be estimated using the Cavalieri estimator (Gundersen and Jensen, 1987):

$$V_{ref} = \sum A \cdot T$$

where $A$ is the sum of the area of the sections/slices analyzed, and $T$ is mean section/slice thickness. $A$ can be estimated using point counting on about 10 consecutive SUR sections/slices from the reference space or by the 2D nucleator.

9.4. Step 3B: Counting podocytes in average glomeruli using a combined optical and physical fractionator

An alternate approach for estimating the number of podocytes per average glomerulus involves a combined optical and physical fractionator, using the equation:

$$N_{pod, avg} = \frac{N_{pod, ref}}{V_{grom, ref}}$$

(11)

where $N_{pod, ref}$ is the number of podocytes in the reference sample (kidney, biopsy) and $N_{grom, ref}$ is the number of glomeruli in the sample. $N_{pod, ref}$ is estimated using:

$$N_{pod, ref} = \frac{1}{SSA} \cdot \frac{1}{HSF} \cdot Q_{pod}$$

(12)

where SSA is the section/slice sampling fraction and HSF is the Q*-weighted height sampling fraction of the sections, equal to $h/h(Q^*)$, where $h$ is the height of the optical dissector defined with a Z-axis analysis and $Q^*$ is the Q*-weighted section thickness (Dorph-Petersen and Lewis, 2011). The number of glomeruli in the biopsy or sample ($N_{grom, ref}$) is estimated at low magnification with physical dissectors (Stein, 1984; Bertram, 1995) using section pairs using:

$$N_{grom, ref} = \frac{1}{SSB} \cdot \frac{1}{Q_{grom}}$$

(13)

where SSB is the section/sampling fraction, $Q_{grom}$ is the number of glomeruli counted in both directions using physical dissectors, and the 1/2 corrects for the fact that glomeruli were counted in both directions. Full details of counting glomeruli with physical dissectors can be found in Bertram (1985) and Nyengaard (1989).

10. Conclusions

Podocyte depletion is considered a significant early step in the development of many forms of glomerular pathology. For a full understanding of podocyte depletion, accurate and precise techniques for estimating podocyte number are required. This paper has described methods for estimating numbers of podocytes in individual (single) glomeruli and in average glomeruli. Adoption of these methods should provide new insights into podocyte depletion in humans and animals, and facilitate the development of diagnostic as well as therapeutic strategies.

Acknowledgements

The authors acknowledge the facilities, scientific and technical assistance of Monash Micro Imaging and the Histology Platform at Monash University, Victoria, Australia. VGP received a Monash Research Graduate School Scholarship and a Faculty of Medicine International Postgraduate Scholarship to support his PhD candidate, this work is funded by the NHMRC of Australia (grant number: 606619) and the Center for Stochastic Geometry and Advanced Bioimaging (CSGB) is supported by the Villum Foundation.

References


Chapter 7

Podocyte number in children and adults:

associations with glomerular size and numbers of

other glomerular resident cells

Declaration for Thesis Chapter 7

Declaration by candidate

In the case of Chapter 7, the nature and extent of my contribution to the work was the following:

<table>
<thead>
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<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Design of experiment, data collection, analysis, and writing and editing of multiple drafts</td>
<td>90%</td>
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The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

<table>
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<th>Name</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%) for student co-authors only</th>
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<tr>
<td>Douglas-Denton, RN</td>
<td>Technical assistance</td>
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<td>Li, J</td>
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<td>Hughson, MD</td>
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<td>Bertram, JF</td>
<td>Experimental design, writing and editing of multiple drafts</td>
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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate’s and co-authors’ contributions to this work.

Candidate’s Signature  

Date: 21-5-14

Main Supervisor’s Signature  

Date: 21-5-14
Podocyte number in children and adults: associations with glomerular size and numbers of other glomerular resident cells

Journal: Journal of the American Society of Nephrology
Manuscript ID: Draft
Manuscript Type: Original Article - Basic Research
Date Submitted by the Author: n/a

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Keywords: glomerular hyperfiltration, podocyte, renal morphology.
Podocyte number in children and adults: associations with glomerular size and numbers of other glomerular resident cells

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Running Title: Podocytes in normal human glomeruli

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Keywords: Glomerular hypertrophy, podocyte depletion, podocyte endowment
Abstract

Increases in glomerular size occur with normal body growth and in a range of pathological conditions. The aim of this study was to determine associations between glomerular size and numbers of glomerular resident cells, with a particular focus on podocytes. Kidneys from 16 Caucasian American males without renal disease, including 4 children (≤3 years old) to define baseline values of early life and 12 adults (≥18 years old), were collected at autopsy in Mississippi, USA. We used a combination of immunohistochemistry, confocal microscopy and design-based stereology to estimate individual glomerular volume (IGV), and numbers of podocytes, non-epithelial cells (NECs, tuft cells other than podocytes) and parietal epithelial cells (PECs). Podocyte density was calculated. Data are reported as median and interquartile range (IQR).

Glomeruli from children were small and contained 452 podocytes (IQR: 335-502), 389 NECs (IQR: 265-498), and 146 PECs (IQR: 111-206). Adult glomeruli contained significantly more cells than glomeruli from children, including 558 podocytes (IQR: 431-746; P<0.01), 1383 NECs (IQR: 998-2042; P<0.0001) and 367 PECs (IQR: 309-673; P<0.0001). However, large adult glomeruli showed markedly lower podocyte density (183 podocytes per x10^6 µm^3) than small glomeruli from adults and children (932 podocytes per x10^6 µm^3; P<0.0001).

Large adult glomeruli contained more podocytes than glomeruli from children and small adult glomeruli, raising questions about the origin of these podocytes. The increased number of podocytes in large glomeruli does not match the increase in glomerular size observed in adults, resulting in relative podocyte depletion. This may render these large glomeruli susceptible to pathology.
Introduction

Chronic kidney disease (CKD) is a global pandemic [1]. There are multiple causes of CKD, and although each cause of CKD shows a particular pathophysiology, most share common features. Arguably, the most significant of these is podocyte injury or dysfunction [2]. Podocytes have a highly specialized structure and a very limited capacity to replicate under normal circumstances [3]. As major components of the glomerular filtration barrier they fulfil a number of important functions, including structural support to capillary loops, synthesis of components of the glomerular basement membrane, synthesis and secretion of several cytokines and growth factors, and immunologic functions [4]. Consequently, podocyte injury comes at a high price for the glomerulus.

In recent years, the podocyte depletion hypothesis has emerged as a unifying concept in the pathogenesis of glomerular disease [5]. Based on multiple observations in animal models [4, 6, 7, 8, 9, 10], podocyte depletion can be defined as “absolute” when it involves a reduction in the total number of podocytes per glomerulus [11, 12]. In a landmark study, Wharram et al [5] showed that a 40% reduction in podocyte number is a direct cause of focal and segmental glomerulosclerosis (FSGS) and thereby long-standing CKD in rats. To date a similar threshold has not been defined in humans.

“Relative” podocyte depletion occurs when the number of podocytes does not keep pace or “match” an increase in glomerular volume or filtration surface area. Glomerular size increases with normal body growth and also prior to and during multiple renal pathologies, including both diabetic nephropathy [13, 14, 15, 16, 17] and FSGS [18, 19, 20, 21, 22, 23, 24]. It has been suggested that an increase in glomerular size, also known as glomerular hypertrophy, is usually a compensatory mechanism that serves to match physiologic demands and sustain renal function [25]. Over the last decade, our group has described multiple factors associated with compensatory glomerular hypertrophy in humans without renal disease, including older age, low nephron number (N_{glom}), obesity, hypertension and African American race [26]. Interestingly, the boundary between compensatory and pathological hypertrophy remains unclear and may be associated with relative podocyte depletion.

Despite the current interest in podocyte depletion, few studies to date have reported total numbers of podocytes in normal glomeruli [27, 28, 29, 30, 31, 32]. This is in part due to the
difficulty in counting podocytes and lack of consensus on how to accurately count them. Lemley et al [33] recently compared five methods for estimating podocyte number and recommended that the design-based disector/optical fractionator method was the optimum method when sufficient kidney tissue was available. This method provides an estimate of the volume of individual glomeruli, as well as the total numbers of each of the resident glomerular cells, including podocytes, endothelial cells, mesangial cells and parietal epithelial cells (PECs) [11]. It thus provides an ideal tool to analyse both absolute and relative podocyte depletion and associations with numbers of other resident glomerular cell types.

In the present study, we used the disector/optical fractionator method to estimate glomerular volume, and numbers of podocytes and other resident glomerular cells in kidneys collected at autopsy in male Caucasian American children and adults. Our findings provide new insights into changes in glomerular cell populations with glomerular hypertrophy in the non-diseased kidney, and suggest that large adult glomeruli have relative podocyte depletion and therefore may be susceptible to the development of pathology.

Results

General demographics

General demographics of the four children are provided in Table 1. Briefly, all four children were 3 years of age or younger, presented no complications during pregnancy and had adequate birth weights for gestational age. Body surface area (BSA) at the time of death ranged from 0.31 to 0.71 m$^2$. Causes of death for all children were not cardiovascular-related and there were no signs of hypertension in these subjects. All of the children presented robust $N_{\text{glom}}$ and $N_{\text{glom}}/\text{BSA}$.

General demographics for the 12 adult subjects are provided in Table 2. The youngest adult was 25 years old, whereas the oldest was 49 years of age. 50% of the adult subjects were either overweight or obese (BMI $\geq$ 25.0 kg/m$^2$, but $< 40$ kg/m$^2$). BSA ranged from 1.45 to 2.64 m$^2$, $N_{\text{glom}}$ from 0.55 to 1.66 million, and $N_{\text{glom}}/\text{BSA}$ from 1.06 to 0.55 million per m$^2$. Birth weight was normal (>2.5 kg and <4.0 kg [34]) in those subjects with available data (n=8). Five of the adults were hypertensive and five had cardiovascular-related deaths.
Glomerular Volumes and Cell Numbers

A total of 96 glomeruli were analysed, comprised of 24 glomeruli from children and 72 from adults. Podocytes were identified according to several criteria (Figure 1A-A’), namely specific cytoplasmic immunostaining for Wilms’ Tumour 1 (WT-1; Figure 1B-B’), presence of major cytoplasmic projections (Figure 1B’), nuclear location outside capillary loops and lack of immunostaining for von Willebrand factor (vWF; Figure 1C-C’). Initially, we intended to use immunostaining for vWF to identify endothelial cells. However, vWF staining was not consistent in all autopsy samples, and we therefore classified all cells on the glomerular tuft that were not podocytes as NECs (the majority of these cells were endothelial and mesangial cells). PECs were identified by their location on Bowman’s capsule.

Glomerular size differs significantly between children and adults

Representative light microscopic images of glomeruli from children and adults are provided in Figures 2A and B respectively. IGV estimates for the 96 glomeruli in the 16 subjects ranked by median IGV are shown in Figure 2C. Considerable variation in glomerular size was present in children, but this was far greater in adults. Adults showed greater aggregated median IGV (Figure 2D; P<0.0001) and IGV variance (Figure 2E; P<0.01) than children. Median IGV in children was 0.41 x10^6 µm^3 (IQR: 0.29-0.64 x10^6 µm^3), and in adults it was 2.42 x10^6 µm^3 (IQR: 1.54-3.27 x10^6 µm^3), a 5.9-fold difference. Figure 2F shows the marked right shift in the adult IGV distribution compared to that in children. The smallest glomerulus observed in children had a volume of 0.21 x10^6 µm^3, while the largest had a volume of 0.89 x10^6 µm^3 – a difference of 0.68 x10^6 µm^3 or a 4.2-fold difference. In contrast, the smallest glomerulus in adults had a volume of 0.76 x10^6 µm^3, while the largest had a volume of 6.91 x10^6 µm^3, a difference of 6.15 x10^6 µm^3, or a 9.1-fold difference.

Numbers of glomerular cells differ significantly between children and adults

Representative confocal images showing the marked differences in glomerular cellularity between children and adults are shown in Figures 3A and 3B. Aggregated data for total numbers of NECs, podocytes and PECs in children (n=24 glomeruli) and adults (n=72 glomeruli) is reported in Figures 3C and 3D; clearly demonstrating the greater variability in adult cell numbers. In adult glomeruli, the median podocyte count was 558 (IQR: 431-746; 3.7-fold), while median NEC and PEC counts were 1383 (IQR: 998-2042; 7.0-fold) and 367 (IQR: 309-673; 10.3-fold), respectively. In children, the median podocyte count was 452 (IQR:
while median NEC and PEC counts were 389 (IQR: 265-498; 4.2-fold) and 146 (IQR: 111-206; 2.8-fold), respectively.

**Numbers of glomerular cells in the context of glomerular hypertrophy**

The estimation of absolute numbers of specific cell types in glomeruli of known volume allowed us to examine relationships between glomerular size and numbers of each cell type. As seen in Figure 4, IGV was directly and strongly associated with the numbers of NECs (aggregated R=0.96, children R=0.83, adult R=0.92; P<0.0001 in each case). In a linear regression analysis of aggregated glomeruli (from children and adults), 92% of IGV variability was explained by numbers of NECs (F: 1084, P<0.0001), predicting an increase of 544 NECs per 10⁶ µm³ of glomerular tuft volume. Similarly, IGV was closely directly associated with PEC number (aggregated R=0.88, children R=0.78, adults R=0.86; P<0.0001 in each case) (Figure 4B). A linear regression model predicted an increase of 141 PECs per 10⁶ µm³ of glomerular tuft volume (R²: 0.79; F: 357; P<0.0001).

The relationship between glomerular size and podocyte number was examined in three ways. Unlike the situation with PECs and NECs, the association between IGV and podocyte number (Figure 5A) was weaker in adults than in children (aggregated R=0.70, children R=0.87, adults R=0.76; P<0.0001 in each case). A linear regression analysis predicted 375 more podocytes per 10⁶ µm³ of glomerular tuft volume in children (R²: 0.66; F: 42.3; P<0.0001), but only 93 more podocytes per 10⁶ µm³ of glomerular tuft volume in adults (R²: 0.63, F: 119; P<0.0001). The adult relationship between number of podocytes and IGV was better fitted in a quadratic model (Figure 5A), with podocyte number plateauing with IGV values greater than about 4 x10⁶ µm³. Because of the great variability in IGV in adults, we also examined the relationships between podocyte number and glomerular size in children, and with tertiles of glomerular size in adults. As shown by Figure 5B, glomeruli from children contained the same number of podocytes as small and medium adult glomeruli (P>0.05). However, large adult glomeruli (tertile 3) contained significantly more podocytes than glomeruli from children and small and medium adult glomeruli (P<0.01). Figure 5C shows that adult glomeruli (all three tertiles) had lower podocyte densities (podocyte number per 10⁶ µm³ of glomerular tuft volume) than glomeruli from children (P<0.01 for small, P<0.0001 for medium and large glomeruli). Large adult glomeruli had a significantly lower podocyte density than small and medium adult glomeruli (P<0.05 for medium and P<0.0001 for small).
Podocyte mismatch
Our findings also suggest a podocyte “mismatch” in adult glomeruli, especially large glomeruli, in terms of cell ratios. Figure 6A illustrates the association between numbers of NECs, podocytes and IGV. The slopes of the lines of best fit for NECs and podocytes were significantly different (F: 550; P<0.0001). While small glomeruli had similar numbers of NECs and podocytes (indeed podocytes slightly outnumbered NECs), in adult glomeruli NECs outnumbered podocytes (Figure 6B), and this was most pronounced in large adult glomeruli in which the NEC/podocyte ration was close to 3:1. These findings suggest a mismatch between NECs and podocytes in the setting of glomerular hypertrophy. In contrast, the trajectories of PECs and podocytes in the context of IGV were very similar (Figure 6C). However, the ratio of PECs/podocyte was significantly higher in adult glomeruli than in children (1:3) (P<0.001 for all tertiles), and in large (1:1) compared to small (1:2) adult glomeruli (Figure 6D). This change in the PEC/podocyte ratio also suggests a mismatch between PECs and podocytes in hypertrophic glomeruli.

Histological evidence of possible sources of podocytes
WT-1+ PECs (also known as parietal podocytes) were found in approximately 90% of glomeruli in both children and adults. These cells were typically found close to the vascular pole and were never observed near the tubular pole (Figures 7A-A’). WT-1+ cells were also occasionally observed in the juxtaglomerular apparatus, including afferent arterioles (Figures 7B-B’).

Discussion
The three major findings of this study were: (1) glomerular size and numbers of glomerular cells vary widely in children and adults without kidney disease, with greater variation in adults; (2) large adult glomeruli contain more podocytes than glomeruli from young children and small adult glomeruli; and (3) these large adult glomeruli have a lower podocyte density that may leave them in a setting of relative podocyte depletion.
Glomerular hypertrophy plays pivotal roles in the development of FSGS [2, 18, 19] and the progression of diabetic nephropathy [35, 36, 37, 38], two of the most common causes of CKD. According to the hyperfiltration theory [25], an imbalance between glomerular mass and physiological requirements drives glomerular hypertrophy as an appropriate compensatory response in order to sustain renal function. We hypothesize that human glomerular hypertrophy is initially a “healthy” compensatory step, that if exaggerated can result in pathology.

Human glomeruli have been reported to increase in size up to 7-fold from infancy to adulthood [39, 40]. The current findings confirm this significant increase in glomerular size from childhood to adulthood – we report a 5.9-fold increase in median glomerular volume between childhood and adulthood - and demonstrate a much greater variability in glomerular sizes in adults than in children. This latter finding suggests that the great variability in IGV found within and between adults is established later in life [41]. We have previously reported that increased IGV median and variance in adult male Americans without overt renal disease is closely associated with low glomerular number [42], older age [43], obesity [44], African American race [45] and hypertension [46]. The present study suggests that glomerular hypertrophy is initially associated with body growth from childhood to adulthood. Further adult hypertrophy occurs in association with aging, nephron loss, hypertension and obesity, all variables that can be present individually or in combination, which reflects the multifactorial nature of glomerular hypertrophy [41].

To date, only a handful of studies have estimated total podocyte number in adult human glomeruli, and these studies focused on pathology. Studies of type 2 diabetes identified absolute podocyte depletion in the early stages of diabetic nephropathy, which was closely related to disease progression [27, 28, 29, 30]. Absolute podocyte depletion was also reported in studies of IgA nephropathy and hypertensive nephrosclerosis [31, 32]. While these studies provided valuable insights into the association between podocyte number and the development and progression of renal disease, our study shows for the first time that podocyte number varies within and between subjects without overt renal disease.

In the present study, median podocyte number per adult glomerulus was 558, with the lowest count being 263 and the highest count being 983 podocytes (3.7-fold range). This finding raises two important questions: (1) when is podocyte number determined; and (2)
what is the optimal number of podocytes required in an adult glomerulus? These questions are considered in turn.

The present findings provide several novel insights into the establishment of adult podocyte number. Firstly, it is clear that glomeruli in young children contain fewer podocytes than adult glomeruli. This suggests that additional podocytes are acquired after early childhood. The origin of these additional podocytes remains unknown, but in recent years there has been great interest on the topic of podocyte replacement in the postnatal period, possibly occurring from progenitor cells. The four children included in this study ranged in age from 3 months to 3 years. This raises the question of what is, and what determines, podocyte endowment at birth. This issue is considered below.

Mature podocytes exit the cell cycle in order to take on a terminally differentiated phenotype and express cyclin-dependent kinase (CDK) inhibitors such as p16^{INK4a}, P21^{Cip1}, P27^{Kip1}, and P57^{Kip2} [47, 48, 49, 50, 51]. After this point, podocytes are considered incapable of undergoing mitosis under normal conditions, although podocyte proliferation occurs in certain pathological conditions such as HIV-associated nephropathy [52, 53]. Podocytes can be induced to proliferate in vitro when cultured from freshly isolated glomeruli [54], but these cells express low levels of many of the podocyte-specific differentiation markers, suggesting that podocytes are only capable of proliferating once they have de-differentiated to a certain degree. Recent evidence suggests that progenitor cells residing in the juxtaglomerular apparatus [55] and Bowman’s capsule (PECs) [56, 57, 58, 59, 60] may give rise to podocytes. Other lines of evidence suggest that new podocytes may arise from bone marrow [61, 62, 63]. In the present study, WT-1+ PECs (parietal podocytes) were observed near the vascular pole in most glomeruli in children and adults, and some WT-1+ cells were observed in the juxtaglomerular apparatus. Whether any of these cells give rise to new podocytes deserves further attention.

There is much interest in whether PECs can give rise to podocytes [64, 65]. Wanner et al [66] recently showed that podocyte generation is mainly active during glomerular development and may occur after acute glomerular injury, but was not observed in aging kidneys or in response to nephron loss. Interestingly, a recent study by Berger et al [67] showed that parietal podocytes (PECs expressing podocyte markers) disappeared from Bowman’s capsule as glomeruli gradually underwent physiological hypertrophy, suggesting that there is a functional “podocyte reserve” that directly differentiates into podocytes on Bowman’s
capsule. The authors proposed that this is explained by the lack of space to accommodate podocytes on the small glomerular tuft of a young child. This hypothesis is supported by our findings that clearly show that glomeruli from young children are small and replete with podocytes (high podocyte numerical density), but have a lower podocyte number than large glomeruli from adults.

A novel method based on flow cytometry of genetically labeled podocytes was used recently to report a 7% increase in podocyte number after acute podocyte injury [66]. This is an interesting approach that has many advantages (i.e. doesn’t rely on antibodies or time-consuming techniques) and provides powerful information. However, we believe that stereological methods may also provide a valuable perspective. For example, parietal podocytes can be labeled by both PEC and podocyte specific transgenic mouse models [67]. Consequently, these cells could have been counted as podocytes by flow cytometry, even if they were sitting on Bowman’s capsule. This issue is not present in stereology-based approaches that take into account cell location within the glomerulus and provide the context of glomerular size. We believe this highlights how histological/stereological approaches, even if laborious and time-consuming, are still valuable tools for the study of podocyte biology. Further studies, especially using unbiased-stereology are urgently needed to quantify the efficiency of glomerular podocyte gain and possible modifiable mechanisms.

To our knowledge, we present for the first time the concept of human podocyte endowment, a term that refers to the number of podocytes we are born with. We believe this concept follows a series of contributions by our group in order to understand the role of nephron endowment in the risk, development and progression of renal disease [26, 42, 68, 69, 70, 71, 72, 73, 74]. Based on the variation in podocyte number in children and adults, we postulate that some glomeruli at birth may be better equipped to deal with glomerular stress than others. It is evident that a more comprehensive analysis of the numbers of podocytes in newborns and young children is warranted, especially to find associations with low birth weight, prematurity and adverse feto-maternal environments. We have commenced studies to answer this critical question.

With the recent interest on the role of podocyte depletion in glomerular pathology, the question arises as to the optimum number of podocytes required in a glomerulus to maintain glomerular health. Wharram et al. [5] defined 40% podocyte depletion as the threshold for established glomerular pathology in a rat model of podocyte injury. To date, a
similar cut-off value has not been reported for the human kidney. While the present study has revealed a 3.7-fold variation in podocyte number per adult glomerulus, we cannot provide a set value for an optimal podocyte number. Despite containing more podocytes, hypertrophic adult glomeruli showed a substantial decrease in podocyte density, which may already mark an increased risk of glomerulosclerosis. The aim of glomerular hypertrophy is to increase filtration surface area in order to sustain renal function [75]. Previous studies in animal models and human tissue have shown that glomerular hypertrophy is closely associated with endothelial or mesangial cell hyperplasia [76, 77, 78]. Therefore the close association between NECs and IGV in the present study was expected. In our study, NECs included endothelial cells [79] and mesangial cells [80]. A recent study by Fukuda et al [81] showed the critical importance of podocyte hypertrophy when compensating during glomerular hypertrophy. This study provided evidence that a possible pathway for pathological glomerular hypertrophy was a mismatch between the volume of podocytes and glomerular volume. Our present study also indicates that in hypertrophic human glomeruli there is a clear mismatch between the increases in the numbers of NECs and podocytes (i.e. the NECs/podocyte ratio is increased in large adult glomeruli). Taken together, we hypothesize that there is evidence of relative podocyte depletion in large hypertrophic glomeruli, and we propose that further studies in pathological tissue will provide the critical evidence in order to define a threshold of podocyte density leading to the development of glomerulosclerosis.

In recent years, PECs have also gained much attention due to their role in the development of glomerulosclerosis [57, 65, 82, 83]. Activated PECs can avidly proliferate, migrate and produce extracellular matrix [84]. To our knowledge, the present study provides the first estimates of total PEC numbers in human glomeruli. The numbers of PECs were highly variable in adult glomeruli (up to 10.3-fold range) and were closely associated with increases in glomerular size. We also report a clear mismatch between numbers of podocytes and PECs in the context of glomerular hypertrophy. The biological significance of higher numbers of PECs in hypertrophic glomeruli remains unclear, but it may be associated with the boundary between physiological and pathological hypertrophy.

While subjects included in this study come from the largest and most comprehensive kidney autopsy series in the world, the present study has several limitations. We acknowledge the inherent limitation of a cross-sectional study design. We emphasize that although the
number of young children included in this study is small, we utilized all available subjects that met our inclusion criteria: males, Caucasian Americans, younger than 3 years of age, adequate birth weight for gestational age, and no intercurrences during pregnancy. These subjects provided a baseline in order to identify changes in glomerular size and cellularity in adulthood. Another limitation of the present study was the inability to present data for endothelial and mesangial cells separately. This was due to the suboptimal vWF immunostaining in some of the autopsy samples.

**In conclusion:** the present study provides evidence of: (1) glomerular hypertrophy in adult glomeruli and a strong direct association between glomerular size and numbers of NECs; (2) higher numbers of podocytes in large adult glomeruli that may reflect both podocyte endowment at birth and podocyte gain in the postnatal period; and (3) relative podocyte depletion in large adult glomeruli that may increase their risk to develop glomerulosclerosis.

**Methodology**

**Subject selection**

Sixteen Caucasian American males were selected for study: four children (≤3 years old) and 12 adults (≥18 years old) without renal disease. The four children had no complications during pregnancy and had adequate birth weights for gestational age. Adult subjects presented different CKD risk factors (older age, low $N_{glomerular}$, high BSA, hypertension or cardiovascular related deaths). Kidneys were obtained at autopsies performed at the University of Mississippi Medical Center, Jackson, Mississippi, USA. Ethical approval was obtained in advance from the Institutional Review Board of the University of Mississippi Medical Center and Monash University Human Research Ethics Committee. Upon collection, kidneys were perfusion-fixed with 10% buffered formalin, bisected and then immersed in 10% formalin for 10 days. Representative kidney blocks from the upper pole and mid-portion of the kidney were embedded in paraffin as previously described [40].

**Measurements**

We estimated IGVs, and absolute numbers of podocytes, NECs and PECs per glomerulus using a design-based stereological approach [11]. This is considered the gold-standard
method for quantification of podocyte number and glomerular volume when sufficient tissue is available [33].

Briefly, 50 serial sections (14 μm thick) were obtained from one paraffin block from each subject for a total of 700 μm of tissue depth. All 50 serial sections were imaged using an Olympus DotSlide system equipped with a 20X objective lens (Olympus, Tokyo, Japan). Thirty glomeruli (10 from each of the outer, middle and inner cortex) per specimen were sampled using physical disectors as previously described [41, 43]. All profiles of these 30 glomeruli were imaged (until there were no more sections through that glomerulus), generally providing between 8 and 16 profiles per glomerulus. Glomerular profiles were labelled with a flag and a unique identifier. These virtual images served as maps to find all profiles of each of the 30 glomeruli during confocal microscopy. The volumes of all 30 sampled individual glomeruli per kidney were estimated using the Cavalieri estimator [85].

Every second section (in adults) and every section (in children) was used for immunohistochemistry. A total of 25 sections per block were rehydrated in 100% ethanol (x 5 mins), 70% ethanol (x 5 mins) and then phosphate buffered saline (PBS; x 5 mins). Sections were then subjected to an antigen retrieval step which involved immersion in 10X citrate buffer for 10 minutes at 90°C (which can be achieved with an automated system, for example DAKO PT Link PT10126). After cooling, slides were washed in PBS and then in 1% filtered Bovine Serum Albumin (BSA) in PBS for 1 hour. Sections were then immunostained using an antibody against Wilms’ Tumor-1 (WT1) antigen (monoclonal mouse anti-human WT1 – DAKO M356101, clone 6F-H2; for podocyte identification) and an antibody against von Willebrand Factor (vWF), which in this case was a polyclonal rabbit anti-human vWF (DAKO A008202; for endothelial cell identification). WT1 (1:50) and vWF (1:200) were diluted in 5ml PBS and 200µl was applied to each slide. After placing a loose coverslip on top of each section, sections were incubated with these antibodies at 4°C overnight. The following day, each section was washed in PBS three times for 5 minutes. Then, goat anti-mouse Alexa® Fluor 488 (1:2,000 – Invitrogen A-11008) and goat anti-rabbit Alexa® Fluor 555 (1:1,000 – Invitrogen A-11001) were added (200 μl per section) and left for 1 hour at room temperature with light-protection. Sections were then washed in PBS three times for 5 minutes. After this step, 200 μl of 4', 6-diamidino-2-phenylindole (DAPI; 1:10,000 – Sigma-Aldridge D9542-10M6) was added to each section and incubated for 10 minutes. Then, sections were washed in PBS three times for 5 minutes and carefully dried. Prolong Gold
(anti-fade mounting medium, Invitrogen P36934) was used for permanent cover-slipping and left for 24 hours to cure. Finally, nail polisher was used to seal the border of the coverslip, and sections were stored at 4°C.

Cells were counted in 96 glomeruli, this being comprised of six glomeruli from each of the 16 subjects. The six glomeruli analysed per subject were the three smallest and the three largest (representing the 10th and 90th percentiles respectively) from the 30 glomeruli per subject used for IGV estimation. The virtual images obtained with the Dotslide workstation were uploaded onto the confocal microscope computer and used as maps to locate all profiles of each glomerulus. Every immunostained section from each of the six sub-sampled glomeruli per subject was imaged with a Leica SP5 laser confocal microscope (Leica Microsystems, Manheim, Germany). Images were obtained using a 40X objective lens (1.25 NA), with a set zoom (1.01), using sequential imaging for 488 nm, 555 nm and UV light. With this configuration, multiple serial optical sections 1µm apart were collected throughout the full thickness of each of the 14µm immunostained sections. Optical disectors were used to sample, and thereby count cells in 8µm out of the 14µm available for each glomerular profile (we did not count cells in a 3µm guard region at the top and bottom of each section as detailed in [11]).

Cells were sampled and counted using optical disectors on the series of 1µm confocal optical images, stacked as a virtual slide and opened using an ImageJ [86] macro that allowed us to turn on and off the three existing channels (blue – nuclei, green – podocyte cytoplasm, red – endothelial cell cytoplasm) and use a counting tool. All nuclei that came into focus in the next 8 optical sections were then counted including those that first came into focus in the final optical section of the disector counting volume. After all newly-appearing nuclei had been identified, we defined podocytes (tuft cells WT-1+ and vWF-), NECs (tuft cells WT-1-) and PECs (cells located on Bowman’s capsule). Cell counts were exported to an Excel spreadsheet and the following formula used to estimate the total number of cells (podocytes, NECs or PECs) in the individual glomerulus (N_{cell, glom}):

\[ N_{cell, glom} = \frac{1}{SSF} \cdot \frac{1}{h/T} \cdot \Sigma Q^- \]

Where SSF represents the section sampling fraction (we used every second section so SSF = 1/2), h/T is the fraction of section thickness used for cell counting (in this case 8µm divided
by the section thickness $T$), and $\Sigma Q^{-}$ is the total number of podocyte nuclei counted using optical disectors.

Podocyte density was calculated by dividing absolute podocyte number per glomerulus by IGV. Ratios were calculated by dividing total numbers of NECs by total numbers of podocytes (NECs/podocyte ratio) and total numbers of PECs by total numbers of podocytes (PECs/podocyte ratio).

**Statistical Analysis**

Data were analysed using GraphPad Prism version 5.04 for Windows (La Jolla California USA) and StataCorp, 2003 (Statistical Software: Release 8; College Station, TX: StataCorp LP). Values are expressed as median ± interquartile range unless otherwise stated. U-Mann-Whitney was used in order to compare variables with skewed distributions and Spearman rank coefficient was used as a measurement of associations. F-tests were applied in order to assess if lines of best-fit were different. Linear regression analysis was performed using IGV as independent variable and numbers of NECs, PECs and podocytes as dependent variables. In all instances a probability (p) value less than 0.05 was considered statistically significant.
References


**Table 1:**

<table>
<thead>
<tr>
<th>Rank</th>
<th>Age (years)</th>
<th>GA (weeks)</th>
<th>BSA (m²)</th>
<th>(N_{\text{glomer}}) (million)</th>
<th>(N_{\text{glomer}}/\text{BSA} \text{ (million/m}^2)</th>
<th>Birth Weight (Kg)</th>
<th>Hypertensive Status</th>
<th>COD</th>
<th>CVRD</th>
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<tbody>
<tr>
<td>Ch1</td>
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<td>35</td>
<td>0.36</td>
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<td>3.08</td>
<td>2.92</td>
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<td>SIDS</td>
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<tr>
<td>Ch2</td>
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<td>0.31</td>
<td>0.84</td>
<td>2.71</td>
<td>3.54</td>
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<td>1.72</td>
<td>2.80</td>
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<td>accident</td>
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<td>0.90</td>
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<tr>
<td>Median</td>
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<td>1.01</td>
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<td>3.23</td>
<td>0%</td>
<td>NA</td>
<td>0%</td>
</tr>
</tbody>
</table>

Legend: Demographic data for 4 male Caucasian American children without kidney disease. 

*Subjects were ranked based on median individual glomerular volume; GA: gestational age at birth; BSA: body surface area; \(N_{\text{glomer}}\): total nephron number; COD: cause of death, SIDS: sudden infant death syndrome, other: haematological, neoplastic or infectious; CVRD: cardiovascular-related death; IQR: interquartile range.*
Table 2:

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<th>BMI (Kg/m²)</th>
<th>BSA (m²)</th>
<th>N&lt;sub&gt;glom&lt;/sub&gt; (million)</th>
<th>N&lt;sub&gt;glom&lt;/sub&gt;/BSA (million/m²)</th>
<th>Birth Weight (Kg)</th>
<th>Hypertensive Status</th>
<th>COD</th>
<th>CVRD</th>
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<td>A1</td>
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<td>18.19</td>
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Legend: Demographic data for 12 adult Caucasian American males without kidney disease. Subjects were ranked based on median individual glomerular volume; BMI: body mass index; BSA: body surface area; N<sub>glom</sub>: total nephron number; COD: cause of death; Other: infectious, haematological, neoplastic, central nervous system related but not cardiovascular, pulmonary; CAD: coronary artery disease, HHD: hypertrophic heart disease; CVRD: cardiovascular-related death; IQR: interquartile range.
Figure 1:

Legend: Podocyte identification. (A) A representative confocal image of a glomerular tuft is shown (merged); using (B) Wilms’ Tumour 1 (WT-1 in green; specific podocyte marker), (C) von Willebrand Factor (vWF in red; specific endothelial cell marker) and DAPI (blue; nuclear marker). The corresponding inserts demonstrate our podocyte identification criteria: (A’): expression of WT-1 in podocyte cytoplasm (B’), the lack of expression of vWF (C’) and their location outside capillaries. Arrowheads show classical podocyte morphology (major projections). Scale bars = 10 μm
**Figure 2:**

Legend: Differences in glomerular size between children and adults. (A) and (B) show representative images of average glomeruli from children and adults respectively using PAS staining; (C) Individual glomerular volume (IGV) values in 16 Caucasian American males; each circle represents one glomerulus, each column represents one subject, with 30 glomeruli per subject; Ch1-4 represent four young children (grey) and A1-12 represent 12 adults (white), ranked by median IGV; (D) Aggregated IGV differences between children and adults; (E) Difference in IGV variance per subject between glomeruli from children and adults; and (F) IGV distributions in children (solid line with grey area) and adults (dotted line). Bars represent median values with interquartile range; ****P<0.0001 and **P<0.01. Scale bars = 50 µm.
Figure 3:

Legend: Total numbers of podocytes, non-epithelial cells (NECs) and parietal epithelial cells (PECs) in children and adults. (A) and (B) show representative confocal images of glomeruli from children and adults respectively with Wilms’ Tumour 1 (WT-1; green), von Willebrand Factor (vWF; red) and DAPI (blue). (C) and (D) show the number of NECs, PECs and podocytes per glomerulus from children and adults; (C) illustrates the variability of each cell type between glomeruli from adults and children; each circle represents the value for a single glomerulus; and (D) provides details of the median, interquartile range (25% and 75% percentiles), minimum and maximum values and the fold range per group; ***P<0.001 and **P<0.01. Scale bars = 100 µm
Figure 4:

Legend: Non-epithelial cells (NECs), parietal epithelial cells (PECs) and glomerular size. (A) Association between IGV and numbers of non-epithelial cells (NECs) in glomeruli from children (grey) and adults (white); (B) Association between IGV and parietal epithelial cells (PECs) in glomeruli from children (grey) and adults (white).
Legend: Podocyte number and glomerular size. (A) Individual glomerular volume (IGV) and numbers of podocytes in children (grey circles) and adults (green circles). Solid lines represent the lines of best fit in children (linear) and adults (quadratic), dotted line shows the beginning of curve plateau at $4 \times 10^6 \mu m^3$; (B) Number of podocytes in children and adult IGV tertiles (small, medium and large glomeruli); (C) Podocyte density in children and adult IGV tertiles (small, medium and large glomeruli); Children: aggregated data from 4 infants (6 glomeruli per subject; n=24 glomeruli); adult IGV tertiles were: tertile 1 (small glomeruli: between 0.76 and $1.72 \times 10^6 \mu m^3$, tertile 2 (medium glomeruli: between 1.74 and $2.86 \times 10^6 \mu m^3$, and tertile 3 (large glomeruli) between 2.94 and $6.91 \times 10^6 \mu m^3$. P values directly over the bars represent comparisons between children (grey bars) and each adult tertile; ****P<0.0001, **P<0.01, *P<0.05, n.s>P>0.05. Bars represent median values with interquartile range.
Figure 6:

**Legend:** Associations between podocytes, non-epithelial cells (NECs) and parietal epithelial cells (PECs). (A) Mismatch between numbers of podocytes (closed circles) and NECs (open circles) in the context of glomerular size (individual glomerular volume – IGV); (B) NECs/podocyte ratio in glomeruli from children and adults by adult IGV tertiles; (C) The link between numbers of PECs (open circles) and podocytes (closed circles) in the context of glomerular size; and (D) PECs/Podocyte ratio in glomeruli from children and adults by adult IGV tertiles. P values directly over the bars represent comparisons between children (grey bars) and each adult tertile; ****P<0.0001, ***P<0.001, **P>0.05. Bars represent median values with interquartile range.
Figure 7:

**Legend: Possible podocyte sources in the human kidney.** (A-A’) Parietal podocytes: parietal epithelial cells expressing Wilms’ Tumour 1 antigen (WT-1) are located close to the vascular pole of a glomerulus (arrowheads); and (B-B’) WT-1+ cells in the juxtaglomerular apparatus, including afferent arterioles (*).
Chapter 8

Discussion and future directions
The general hypothesis tested by the experiments described in this thesis was: “Glomerular hypertrophy in subjects without renal disease is multifactorial, compensatory and associated with relative podocyte depletion” (Figure 8). Each experimental chapter of this thesis addressed a component of this general hypothesis. The findings are now discussed.

8.1 The classical view of glomerular hypertrophy

Glomerular hypertrophy is conventionally viewed as a compensatory mechanism in response to a reduction in renal mass that can be developmentally programmed or acquired \[1\]. This was first proposed by Brenner et al. [2] almost 30 years ago in animal models of renal mass...
reduction as part of the “hyperfiltration theory”. In principle, remaining glomeruli should undergo a process of hyperfiltration, or an increase in their filtration load. The anatomical correlate of glomerular hyperfiltration is an increase in filtration surface area, also known as glomerular hypertrophy.

Glomerular hypertrophy occurs in humans under a variety of settings. These include: (1) renal mass reduction (for example, in kidney donors [3-5]); (2) congenital abnormalities (such as in oligomeganephronia [6] and unilateral obstructive uropathies [7]); and (3) following development in an adverse feto-maternal environment leading to reduced nephron endowment (as is observed in Australian Aborigines [8]). Furthermore, glomerular hypertrophy has been associated with multiple pathologies, including obesity-related glomerulopathy [9], mesangial proliferative glomerulonephritis [10], FSGS [11], and diabetic nephropathy [12]. In such studies however, it is hard to determine if the glomerular hypertrophy is due to glomerular loss, or if there are other factors involved.

8.1.1 Glomerular hypertrophy is multifactorial

Glomerular hypertrophy has been independently associated with multiple CKD risk factors, including older age [13], lower nephron number [14], obesity [15], low birth weight [15], and African American race [16] in subjects without renal disease. However, the analysis of independent contributors to glomerular hypertrophy tends to over-simplify more complex processes, principally because subjects rarely present with only one risk factor. The findings presented in Chapter 2 provide evidence that glomerular hypertrophy is in fact multifactorial, with a combination of factors driving different levels of hypertrophy, including older age, obesity and hypertension. As a secondary endpoint, analyses in Chapter 2 suggest that the technique used to estimate glomerular volume, namely individual glomerular
volume (IGV), could be further optimised for future clinical applications. Currently, the method is based on systematic sampling and measurement of 30 glomeruli, 10 glomeruli per cortical zone (superficial, juxtamedullary and middle). The limited number of available glomeruli and the cortical area represented in biopsies, which typically corresponds to middle and superficial cortical zones only, restrict the application of IGV to clinical samples. Using a statistical simulation, it was found that zonal sampling and a reduction of sampled glomeruli (from 30 to 9) did not affect the average glomerular volume or the associations observed with other parameters, such as nephron number. However, previous studies have shown that CKD risk factors, such as low \( N_{\text{glom}} \), are not only associated with increases in average glomerular volume, but are also closely associated with greater IGV variability within subjects. It is worth noting that the more glomeruli are measured, the greater the variation likely to be detected.

**Chapter 3** provides further evidence of the multifactorial nature of glomerular hypertrophy. The findings show that glomerular hypertrophy is a feature of hypertensive nephropathy in both Caucasian and African Americans. More importantly, glomerular hypertrophy is exacerbated in African Americans, particularly in the context of older age, lower nephron number, hypertension and obesity. These findings further support the hypothesis that multiple factors can produce glomerular hypertrophy in one individual.

While the histological measurement of IGV could theoretically be applied to clinical biopsies, the ideal method to estimate glomerular volumes would be non-invasive, reliable and would estimate the volume of all glomeruli within a kidney. In this regard, the first steps towards estimating glomerular volumes in whole *ex vivo* kidneys using MRI have recently been described [17, 18]. The method employs cationized ferritin to bind to the GBM and provides visualization of every glomerulus in rat kidneys. **Chapter 5** provides the first MRI report of
total glomerular number, mean glomerular volume and the glomerular size distribution in \textit{ex vivo} human kidneys. It seems likely that imaging-based methods to measure glomerular number and the glomerular size distribution \textit{in vivo} will be available in the not too distant future. The development of such methods will provide an ideal platform for longitudinal studies on human nephron number and glomerular hypertrophy.

\textbf{8.1.2 Glomerular hypertrophy is compensatory}

The inverse association between human $N_{\text{glom}}$ and glomerular volume is now well established. This relationship has been described in Danes [19], Caucasian and African Americans [20-22], Australian Aborigines [23] and African Senegalese [24]. However, an important question remains - do all people with low $N_{\text{glom}}$ develop glomerular hypertrophy? \textbf{Chapter 4} addresses this question by exploring associations between glomerular hypertrophy and body size in the context of $N_{\text{glom}}$ (Figure 9). The study design was based on the principle that females have smaller body sizes than males in our American cohort. Using a precise matching strategy we showed a strong association between glomerular hypertrophy and a combination of low $N_{\text{glom}}$ and large body size. This was in contrast to subjects with low $N_{\text{glom}}$ and low body size, who had smaller glomeruli than subjects with high $N_{\text{glom}}$. These findings suggest that in subjects without renal disease, glomerular hypertrophy may be a compensatory growth response driven by an imbalance between glomerular mass and physiological demands.

\textbf{Chapter 4} also provided the first IGV data for female Americans. The findings strongly suggest that sex-associated differences in glomerular size are mainly driven by differences in body size. While the study described in \textbf{Chapter 4} has several limitations, including small sample size and the cross-sectional nature of the study, the findings hold significant clinical
value. For example, LBW has gained significant relevance as a risk factor for CKD, mostly due to its association with low $N_{\text{glomer}}$. Data from the present study suggest that patients who present with symptoms of CKD who were born with LBW (or in this case with low $N_{\text{glomer}}$) may benefit from lower adult body size, lower BMI, and perhaps weight loss. Robust prospective clinical data are urgently needed to assess whether body size modulation can in fact prevent and/or reverse glomerular hypertrophy. Again, a safe non-invasive method for measuring glomerular size in vivo would be very advantageous in such studies.

![Diagram](image)

**Figure 9: Is glomerular hypertrophy compensatory?** This schematic shows the working hypothesis that an imbalance between nephron number and body size can drive glomerular hypertrophy.

### 8.1.3 Is there a safe limit for glomerular hypertrophy?

The findings from the studies described in Chapters 2, 3 and 4 suggest that human glomerular hypertrophy is multifactorial and compensatory. It is important to remember that these studies focused on glomeruli without pathological changes in subjects without
renal disease. However, many of these subjects would have CKD risk factors, which raises the issue as to whether some of the observed glomerular hypertrophy represents pre-disease events. This reminds us that the limit or threshold between physiological and pathological glomerular hypertrophy remains unclear.

Excellent examples supporting the existence of such a threshold have been described in studies of human biopsies and animal models of disease. Young et al. [25] measured glomerular volumes in renal biopsies of Australian Aborigines with FSGS and showed that glomeruli enlarged up to the point of Grade 3 sclerosis, but were markedly shrunken by Grade 4 sclerosis when capillary collapse and sclerosis were most advanced. Hughson et al. [26] confirmed a similar biphasic pattern of glomerular size in FSGS patients. A similar conclusion can be drawn from rodent models of renal mass reduction. For example, adult rats subjected to uninephrectomy sustain renal function by undergoing hyperfiltration and glomerular hypertrophy. However, after 12 weeks [27, 28], further glomerular hypertrophy cannot continue, glomerulosclerosis is first observed, and renal function declines.

This link between glomerular hypertrophy and the development of glomerulosclerosis suggests that the measurement of glomerular volume could be useful as an outcome predictor in human renal biopsies. Danilewicz et al. [29] and Fogo et al. [30] showed that glomerular hypertrophy is a valuable predictor of unfavourable evolution of FSGS, and that measurement of glomerular size may be of use in differentiating between patients with minimal change disease and those who will subsequently develop FSGS.

It is also important to note that most studies of human glomerular volume have relied on the measurement of a single cross-section from each glomerulus. This is problematic for at least three reasons. First, the estimation of glomerular volume from a single section is very much reliant on the location (equatorial section or polar section) of that section through the
glomerulus. Secondly, if a glomerulus contains a sclerotic segment, a single section may or may not section through that segment. Therefore, a glomerulus may incorrectly be classified as non-sclerotic even when it contains a sclerotic segment. And finally, a glomerulus may contain a large segment of sclerosis, but a single section might only contain a small amount of that sclerosis. Therefore, a glomerulus may be graded as having Grade 1 sclerosis, when in fact it might have Grade 2 or 3. Interestingly, Remuzzi et al. [31] determined that cross-section analysis of glomerulosclerosis greatly underestimates the incidence of sclerosis, which can be resolved by using a three-dimensional approach. Unfortunately, like stereology, three-dimensional approaches are tedious, time-consuming and expensive, and are therefore usually limited to research scenarios where sufficient tissue is available. Renal biopsies pose a particular challenge due to their central role in clinical diagnosis and research.

8.2 Podocyte depletion

Podocyte depletion can be defined as absolute (reduction in the number of healthy podocytes per glomerulus) and relative (reduction in podocyte density per glomerulus). Both absolute [32] and relative [33] podocyte depletion have been linked to the development of glomerulosclerosis in rodents, but prior to the experiments described in this thesis there was little information on variation in podocyte number in normal human glomeruli, or evidence of podocyte depletion in association with human glomerular hypertrophy.

Podocyte depletion is most commonly assessed by counting the number of podocyte nuclei per glomerular cross-section or by using model-based stereological methods such as the method of Weibel and Gomez [34]. Both of these approaches are flawed, the former because it does not provide the absolute number of podocytes per glomerulus, and the
latter because it requires assumptions of podocyte nuclear size and shape. To the extent that these assumptions are incorrect, the estimates of podocyte number will be biased. This difficulty, and associated controversy in podocyte counting was considered recently in an evaluation of five methods for counting podocytes. Lemley et al. [35] concluded that a design-based stereological method known as the disector/fractionator method was the preferred method when sufficient tissue was available, as for example in the case of whole autopsy kidneys. The development of this technique is described in detail in Chapter 6. The method involves serial sectioning of paraffin-embedded tissue, immunolabelling of podocytes, laser confocal microscopy and the application of stereological principles, including the physical disector/Cavalieri estimator for glomerular volume and the disector/fractionator estimator for numbers of glomerular cells, including podocytes. This is the recommended method for the quantification of podocytes in glomeruli of known volume because: (1) it does not require geometric assumptions of glomerular or podocyte nuclear size or shape; (2) it provides quantitative data of both absolute and relative podocyte depletion; and (3) it provides data for absolute numbers of glomerular cells (podocytes, NECs, PECs), as well as cell densities and cell ratios. However, the method also has some limitations: (1) it is time-consuming and therefore expensive in terms of personnel; (2) it requires substantial tissue, which limits its application to renal biopsies; and (3) it relies on immunolabelling to identify podocytes.

The latter point is particularly important because it determines the reliability of the podocyte counting method used in this thesis. Briefly, WT-1 is considered an excellent epithelial cell marker in many organs, including the pleura, spleen, heart, foreskin, endometrium and kidney [36]. In the kidney, WT-1 has been consistently used as a specific podocyte marker [37]. There is evidence in the literature of WT-1 isoforms being present in
both the nucleus and cytoplasm of multiple cell types [38]. While the cytoplasmic expression of WT-1 in podocytes was surprising in the present study, Su et al. [39] had previously described this localization pattern in human podocytes using the same antibody. Appendix 1 shows evidence of co-expression of WT-1 and Synaptopodin, a marker of the actin cytoskeleton in podocyte foot processes and consistent cytoplasmic WT-1 expression in human tissue from autopsies, nephrectomies and biopsies.

Recent evidence shows that WT-1 can also be expressed by PECs [40] as well as cells of the juxtaglomerular apparatus [41]. This represents a problem if the podocyte counting method relies solely on WT-1 immunolabeling. However, as detailed in Chapters 6 and 7, several criteria other than WT-1 labeling were used to identify podocytes in the present study. These criteria included lack of vWF expression, nuclear location outside capillaries, and morphologic features of a podocyte, namely major cytoplasmic projections. In addition, the WT-1 antibody successfully labelled podocytes in nephrectomy, transplant and biopsy samples. Taken together, the evidence presented above convincingly demonstrates that the WT-1 labeling of podocytes in this thesis was both valid and specific. Nevertheless, efforts to improve the efficiency of this method are urgently needed, and suggestions for such developments are provided in Section 8.3.4.

8.2.1 The classical view of podocyte depletion: absolute podocyte depletion

Absolute podocyte depletion is generally defined as the loss of healthy mature functional podocytes [42]. Absolute podocyte depletion is considered a significant event in the development of glomerulosclerosis [43]. While findings from numerous studies have suggested the pivotal role of podocyte injury and loss in the development of sclerosis, a landmark study in rats by Wharram et al. [32] showed that loss of 40% or more of podocytes
lead to the development of glomerulosclerosis and progressive kidney disease. A similar limit has not yet been established in humans, but the findings from the studies presented in Chapter 7 provide the first steps towards identifying such a threshold.

The first main finding from the studies described in Chapter 7 is that there is considerable variation in the total number of podocytes per glomerulus in both children and adults. A 3.7-fold range in podocyte number per glomerulus was observed in adults, and a 2-fold range was observed in children. These findings suggest that variation in human podocyte number may in part be established during glomerular development, and possibly be exacerbated by events that occur after birth. These findings, albeit in a total of 12 adults and four children, not only provide the most comprehensive baseline values to date for human podocyte number and variation, but also provide unique insights into the previously unexplored topic of podocyte endowment. This latter concept raises the issue of whether some children are born with fewer podocytes (low podocyte endowment), and whether this increases their susceptibility to CKD in later life. This is certainly a topic for future investigation (see Section 8.3.3).

8.2.2 Glomerular hypertrophy and relative podocyte depletion

Podocyte depletion can also be defined as relative, when the number of available functional podocytes does not match the increase in glomerular volume or filtration surface area. This type of podocyte depletion was recently studied by Fukuda et al. [33] who showed that increases in body weight in mice, leading to increases in glomerular volume, were not matched by increases in podocyte number or podocyte volume, eventually leading to the development of glomerulosclerosis. The findings in Chapter 7 highlight how podocyte density decreases significantly in adulthood, especially in hypertrophic glomeruli. We
postulate that these large glomeruli may be at a higher risk for the development of glomerulosclerosis, and suggest that podocyte density is an important parameter to assess in future human studies in order to define the threshold between physiological and pathological glomerular hypertrophy.

8.2.3 Can glomeruli acquire more podocytes after birth?

There is currently much controversy in the nephrology community on the topic of podocyte regeneration. For decades, podocytes have been thought to be terminally differentiated cells with very limited capacity for replication [44]. This is still the dogma in nephrology, however recent evidence suggests that new podocytes might be derived in the adult kidney from PECs [45] and from cells in the juxtaglomerular apparatus [41].

Recent findings from Pichaiwong et al. [46] showed that leptin replacement, but not inhibition of the RAAS, resulted in near-complete reversal of both functional and structural measures of advanced diabetic nephropathy, including podocyte number, in a leptin-deficient BTBR ob/ob mouse, a model of advanced diabetic nephropathy. The authors identified an increase in the numbers of PECs expressing podocyte markers (parietal podocytes) after leptin treatment, and suggested parietal podocytes may be a source of podocyte gain. Interestingly, similar observations have been reported in a mouse model of FSGS with recovery of podocyte number following corticosteroid treatment and de novo expression of podocyte markers in PECs [47]. The latter was also found following treatment with retinoic acid in a rat model of membranous nephropathy and a mouse model of FSGS [48], and in a rat model of aging nephropathy [49].

During glomerular development, and in adolescent rodents, PECs actively migrate to the glomerular tuft where they express podocyte markers and locate in a position resembling
podocytes [50]. However, when glomerular maturity is achieved and podocytes express markers of cell differentiation such as nephrin, this migration slows. Once glomerular maturation is complete, and when rodents become adults, PEC migration stops. Based on these findings, it has recently been proposed that early in glomerular development, a subpopulation of PECs commits to the podocyte lineage (also known as parietal podocytes), but due to lack of space in young and small immature glomeruli, these cells are stored at the vascular pole until sufficient space is available to allow migration and integration into the glomerular tuft as podocytes [40]. If this proposition is correct, a setting of glomerular hypertrophy might be expected to drive migration of PECs to the glomerular tuft during early life and adolescence, when significant body growth occurs (Figure 10).

![Figure 10: The contribution of PECs towards podocyte number.](image)

The findings in Chapter 7 support this hypothesis in humans. Glomeruli from children contained significantly fewer podocytes than glomeruli from adults. This was especially the case when glomeruli from children were compared with large adult glomeruli. It is possible
that the high numbers of podocytes observed in large adult glomeruli may in part be due to the accumulation of new podocytes during periods of normal body growth following the completion of glomerulogenesis. While the present findings support the possibility of podocyte gain after birth, the origin of these additional cells is not clear and goes beyond the scope of this study.

While PECs expressing WT-1 were clearly observed in several glomeruli from both children and adults in the present study, it is possible that these cells are not the only source of podocyte gain. In the present study, several cells in the juxtaglomerular apparatus also expressed WT-1, as has been previously described [41]. It is possible that these cells are renin-expressing cells that are able to migrate into the tuft in order to contribute to the podocyte pool. Recent publications by Pippin et al. [41] from studies in mice suggest that a subpopulation of cells of renin-lineage move to the glomerular tuft and differentiate into functional podocytes; however, their contribution is not sufficient to restore normal podocyte number.

Overall, studies from a handful of recent studies suggest that more than one mechanism may contribute to podocyte gain following birth in humans, but much more research is required before this contentious and important issue is resolved. Nevertheless, with the generally accepted pivotal role of podocyte depletion in the development of multiple causes of CKD, it can be said that podocyte gain in adult humans in many cases fails to prevent the onset and progression of disease.
8.3 Future directions

8.3.1 Glomerular hypertrophy from the bench to the clinic

Chapters 2, 3 and 4 clearly highlight the value of glomerular volume measurements and their potential clinical applications. Currently, methods for the reliable non-invasive measurement of glomerular number and volume are not available. However, the first steps towards the development of such methods have recently been described. Publications by Heilmann et al. [17] and Beeman et al. [18] provided the foundation for clinical translation in the assessment of glomerular volume by using a combination of cationized ferritin and MRI to count and size every glomerulus in the ex vivo rat kidney. Furthermore, Chapter 5 is the first study using MRI to quantify glomerular number and size in ex vivo human kidneys. As an ex vivo approach, MRI could be useful in the context of renal transplantation in order to assess glomerular mass before implantation in the recipient. While further steps are needed in order to turn this approach into an in vivo diagnostic tool, MRI appears to have the most realistic potential for immediate clinical translation in prospective longitudinal clinical studies of glomerular number and hypertrophy.

8.3.2 The threshold between physiological and pathological glomerular hypertrophy

Throughout this thesis, especially in Chapters 2, 3 and 4, significant evidence is provided to support the concept that glomerular hypertrophy is a physiological process in subjects without renal disease. However, the findings in Chapter 7 suggest that glomerular hypertrophy, while physiological, is also closely associated with a reduction in podocyte density, which raises a “red flag”. The latter may render these glomeruli susceptible to glomerulosclerosis. Efforts to define the limit between physiological and pathological
glomerular hypertrophy should be directed at future comprehensive assessment of podocyte density in health and disease.

To our knowledge, **Chapter 7** provides the first report of numbers of PECs in the normal human kidney. Recent evidence suggesting a role for PECs in podocyte gain has been described and analysed in several chapters of this thesis. However, PECs can undergo a process of activation, which consists of four main features: *de novo* expression of the surface marker CD44, *de novo* production of extracellular matrix, increased proliferative capacity and increased migration capabilities [52, 53]. When activated, PECs can adhere to areas of denuded GBM (usually due to podocyte injury) and create “bridges” or adhesions between Bowman’s capsule and the GBM, an important early pathological event leading to glomerulosclerosis [54]. While PEC activation is a well-accepted pathway leading to sclerosis, the molecular mechanisms driving this process are poorly understood. In **Chapter 7**, a close association between numbers of PECs and glomerular size is reported. In theory, the higher the numbers of PECs present in Bowman’s capsule, the greater the numbers of cells that if activated could drive a pathological sequence. It is in this context, that glomerular hypertrophy, podocyte depletion and the activation of PECs may operate together in order to initiate pathology.

PDGF plays a key role in driving glomerulosclerosis in the context of experimental glomerular hypertrophy [55]. PDGF is produced by endothelial cells and has several target cell populations in the glomerulus, including mesangial cells and PECs [56]. Multiple studies, including Nagata et al. [28] and Fukuda et al. [33] have shown that during glomerular hypertrophy, podocytes undergo hypertrophy in order to adequately cover the amplified filtration surface area and cope with a marked reduction in podocyte density (relative
podocyte depletion). Podocyte hypertrophy may alter the filtration barrier allowing the passage of certain molecules [57-59].

It is worth noting, that in Chapter 7, features of adult hypertrophic glomeruli are described, including: increased numbers of endothelial and mesangial cells (producers and effectors of PDGF), reduced podocyte density (possibly linked with podocyte hypertrophy and thereby podocyte stress), and increased the number of PECs (which may sense podocyte stress, and could be activated at any time). PECs have a receptor for PDGF [55] and evidence suggests that PDGF overexpression can lead to the development of cellular crescents, a classical pathological feature of PEC activation [60]. Once PDGF crosses the filtration barrier, it can potentially initiate PEC activation, thereby facilitating the development of glomerulosclerosis in a glomerulus that is under major stress. This hypothesis requires further testing, ideally in transgenic animal models that allow PDGF tracking and overexpression, as well as in animals with reduced PDGF activity, in combination with reliable cell counting methods. This integrative view of glomerular hypertrophy may provide the foundation for future therapeutic strategies.

8.3.3 Podocyte endowment: a new target in developmental biology

To our knowledge, the term “podocyte endowment” has not previously been used in adult or paediatric nephrology. However, it is well accepted that mature podocytes are highly specialized cells with limited capacity for replication. Therefore, the numbers of podocytes at birth or podocyte endowment may well reflect the podocyte pool available throughout the lifespan. The podocyte counts for the four children presented in Chapter 7, most likely constitute the most comprehensive report to date of podocyte number in children. Podocyte
number per glomerulus varied 2-fold in the children, from 302 to 634, raising the question of how podocyte endowment is regulated.

The role of the feto-maternal environment in the regulation of nephron endowment and thereby the risk of subsequent kidney disease is now a well-accepted principle [61]. In animal studies, a range of perturbations to the feto-maternal environment result in low nephron endowment [62, 63], including exposure to: maternal nutrient restriction, natural or synthetic corticosteroids, use of certain antibiotics, maternal ethanol ingestion, and maternal hyperglycaemia. The possibility that podocyte endowment could be affected by perturbations to the feto-maternal environment is an interesting one, and given that podocytes have a very limited capacity for replacement in the adult kidney, is a promising topic for future research.

8.3.4 Quantifying podocyte gain: efficiency and possible modulators

Throughout this thesis, the importance of reliable techniques for quantitation of glomerular volume and podocyte number has been highlighted. However, it is not common practice to apply design-based stereology when assessing these variables, principally because stereology is time-consuming and expensive in terms of labour [64]. Efforts to improve the time- and cost-efficiency of these techniques would potentially be of great value for future studies. It is theoretically possible to modify the immunolabelling protocol used in the present study for application to thick kidney slices (i.e. 2 mm) and image whole glomeruli using laser confocal microscopy. This approach, combined with 3D rendering software (such as Drishti® and Imaris, Bitplane) could significantly reduce analysis time thereby facilitating the use of unbiased stereology. Depending on the available antibodies, several variables
could be assessed, including glomerular volume, numbers of podocytes, podocyte density and possibly podocyte volume.

On the foundation of valid quantitative techniques, the measurement of podocyte loss and gain in transgenic animals in which cell lineages can be unambiguously traced would be an important next step. While the current evidence suggests that PECs can migrate from Bowman’s capsule to the glomerular tuft in growing glomeruli in young mice, neither the efficiency of this process nor its molecular regulation is well understood. Similarly, the efficiency of cells of the renin lineage to contribute to podocyte turnover requires further assessment. Overall, the future combination of reliable quantification techniques and transgenic animal models is essential to answering the important fundamental questions related to podocyte endowment, depletion and gain, and their associations with glomerular pathology.
References


Appendix 1

Further validation of selected podocyte marker
Figure 1:

Legend: Further validation of Wilms’ Tumour 1 (WT-1) as a podocyte marker in our autopsy series. Panels A, B and C show Synaptopodin (SNP) and WT-1 labeling in glomerular cells with podocyte morphology, including major projections and location outside the capillaries. Panels D-F show glomerular labeling of von Willebrand factor (vWF) and WT-1, which clearly show that those cells expressing cytoplasmic WT-1, not only show a podocyte morphology, but do not express markers of other glomerular cells, such as vWF, a well-characterized endothelial cell marker. Arrows follow one cell as an example for each sequence.

Figure 2:

Legend: Cytoplasmic Wilms’ Tumour 1 (WT-1) is consistently observed in human samples from autopsy, nephrectomy and biopsies. vWF: von Willebrand factor and DAPI: 4’,6-diamidino-2-phenylindole.