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PRETERM BIRTH: EFFECTS ON RENAL DEVELOPMENT AND FUNCTION

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

Doctor of Philosophy

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SUMMARY

Worldwide, approximately 10% of all births are preterm (defined as delivery prior to 37 completed weeks of gestation). Preterm birth is the leading cause of perinatal mortality and morbidity, which may be attributed to the structural and functional immaturity of a number of organ systems after birth. In the kidney, nephrogenesis (the development of nephrons) is ongoing in late gestation, with the final complement of nephrons normally only formed by 32-36 weeks gestation. Therefore, in infants born preterm, the development of the kidney is often still ongoing at the time of birth; it is unknown, however, what effect this may have on renal development. In addition, the preterm infant may be exposed to a number of factors in the extrauterine environment (such as supplemental oxygen therapy and exposure to nephrotoxic medications) that may further influence renal development, and may also lead to renal injury. It was therefore hypothesised that preterm birth (and/or related factors in the postnatal care of the preterm neonate after birth) would adversely affect renal development and function in the neonatal period. To address this hypothesis, studies of structural and functional renal development in human preterm neonates, as well as in preterm baboon, preterm lamb and neonatal mouse models, were undertaken.

The aims of the first two experimental studies reported in this thesis were to determine the effects of preterm birth on renal function (Chapter 2) and renal morphology (Chapter 3) in human neonates. The preterm neonatal kidney was shown to be functionally immature during the first month of life, with a lower rate of creatinine clearance and higher sodium and protein excretion than neonates born at term. Renal maturity was the major determinant of renal functional capacity; however, pathological proteinuria and high urinary neutrophil gelatinase associated lipocalin (NGAL) levels suggested that postnatal renal injury had also occurred in some neonates. The results of Chapter 3 demonstrated that nephrogenesis was ongoing in the extrauterine environment following preterm birth. Evidence of accelerated renal maturation, glomerular hypertrophy, and abnormal glomerular morphology suggest, however, that the ongoing nephrogenesis may have been impaired. Dilation of glomerular capillaries was also observed in the lamb kidney following preterm birth (Chapter 4); if this persists, it is probable that glomerular

hypertrophy may in the long term result in glomerulosclerosis and subsequent nephron loss.

In Chapters 5 and 6 of this thesis, specific factors commonly involved in the postnatal care of the preterm neonate were investigated as possible causes of impaired renal development. As reported in Chapter 5, exposure to moderate hyperoxia (65% O_2) did not appear to have any effect on postnatal nephrogenesis in a neonatal mouse model; there was no change in the maturation of glomeruli, and nephron endowment was within the normal range. In early adulthood, however, glomerular hypertrophy was evident in the hyperoxia-exposed animals, which suggests the possibility of an underlying impairment in vascular development and/or injury. As shown in Chapter 6, early postnatal treatment with the nephrotoxic medication ibuprofen (commonly used in the treatment of a patent ductus arteriosus in preterm neonates) resulted in a significant reduction in nephrogenic zone width; this may imply the early cessation of nephrogenesis.

Overall, the findings of this series of studies suggest that the nephrogenic potential of the preterm neonate is not being reached after birth. Ultimately, this may result in a deficit of functional nephrons in the preterm kidney, which is likely to have an adverse effect on both the short and long-term renal health of individuals born preterm. It is essential that future research be focused on the identification of factors that adversely influence development of the preterm kidney, in order to develop strategies that can be implemented in the neonatal intensive care setting to optimise postnatal renal development.

GENERAL DECLARATION

In accordance with Monash University Doctorate Regulation 17 / Doctor of Philosophy and Master of Philosophy (MPhil) regulations, the following declarations are made:

I hereby declare that preliminary results from the experimental studies reported in Chapters 2 and 3 of this thesis were previously documented in a Ph.D. thesis by Dr. Lina Gubhaju (*The effect of preterm birth on the kidney,* Monash University, 2009). As continuation of these previous studies, in this thesis additional neonates have been added to the experimental groups, further analyses have been undertaken, and the studies have been completed for publication. Along with Dr. Gubhaju, I have written the manuscripts relating to this work. In addition, the animal studies described in Chapter 5 were previously documented in a Ph.D. thesis by Ms. Megan O'Reilly (*The effects of neonatal inhalation of hyperoxic gas on airway development and lung function in later life,* Monash University, 2012). In this thesis, I conducted analyses of the kidneys of mice that had been previously generated in Ms. O'Reilly's animal studies.

Except for that declared above, 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 2 original papers (Chapters 3 and 6) and 2 review papers (included in Chapter 1) that have been published in peer reviewed journals, 1 published book chapter (included in Chapter 1), 1 submitted paper under revision (Chapter 5) and 2 unpublished manuscripts in a form ready for submission (Chapters 2 and 4). The core theme of this thesis is the effects of preterm birth on renal development and function. 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 Associate Professor M. Jane Black.

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 Chapters 4, 5

and 6, the animal studies were performed by our collaborators, and I conducted the analyses of the kidneys of these animals.

In the manuscripts included in this thesis (Chapters 1-6), my contribution involved the following:

Thesis chapter	Manuscript Title	Publication status	Nature and extent of candidate's contribution
1	Preterm birth and the kidney: Implications for long-term renal health *(Review)*	Published	Wrote some sections of manuscript Total contribution: 20%
1	Effects of preterm birth on the kidney *(Book Chapter)*	Published *(eBook)*	Wrote 'renal function' section of manuscript Total contribution: 35%
1	Stereological assessment of renal development in a baboon model of preterm birth *(Review)*	Published	Wrote majority of manuscript Total contribution: 85%
2	Assessment of glomerular and tubular function, and a marker of renal injury, in preterm neonates during the first month of life	Unpublished	Performed ~60% of experimental work, all data analysis, and co-authored the manuscript Total contribution: 50%
3	Accelerated maturation and abnormal morphology in the preterm neonatal kidney	Published	Performed half of the experimental work, majority of data analysis, and co-authored manuscript Total contribution: 50%
4	Glomerular capillary length and surface area in the preterm lamb kidney	Unpublished	Performed all experimental work (except for animal studies), all data analysis, and wrote manuscript Total contribution: 70%
5	Neonatal hyperoxia leads to glomerular hypertrophy in the adult mouse kidney	Submitted *(Under revision)*	Performed majority of experimental work (except for animal studies), all data analysis, and wrote manuscript Total contribution: 60%
6	Effects of ibuprofen treatment on the developing preterm baboon kidney	Published	Performed majority of experimental work (except for animal studies), all data analysis, and wrote manuscript Total contribution: 60%

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: [redacted] **Date:** 30/04/12

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The work of Dr. Lina Gubhaju was also instrumental in establishing the 'preterm birth and the kidney' research projects, and I am grateful for all of the training, encouragement, and advice she has provided me. It has been a pleasure to work with Jane, Lina and all members of the Black lab; in particular, my gratitude goes to Kimberley Ong, Laura Stamp and Kom Yin for their assistance with my research studies, and Jonathan Bensley for knowing everything about everything, and for keeping me entertained! Thank you to everyone in the Department of Anatomy and Developmental Biology who supported my work, especially the head of department Professor John Bertram. I also much appreciated the enthusiasm and assistance of Sue Connell, Julie Hickey, Ian Boundy and Stef Tombs, who trained me in resin and histology laboratory techniques.

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Disease (University of Queensland) during my candidature, where Professor Wendy Hoy and Ms. Susan Mott provided valuable assistance with statistical analyses.

The animal models (baboon, lamb and mouse) used in these studies were generated by other laboratories: thank you firstly to Professor Brad Yoder (Department of Pediatrics, University of Utah, USA) for his indispensable help on all of our preterm baboon studies; the baboon studies reported in this thesis were performed at the Southwest Foundation for Biomedical Research (Texas, USA) through the work of Professor Donald McCurnin, Professor Steven Seidner and also Professor Ronald Clyman (University of California, San Francisco, USA). Professor Kurt Albertine and Ms. Mar Janna Dahl (University of Utah, Utah, USA) developed the preterm lamb model, and were of great assistance in shipping the kidneys to Australia. I am also grateful to Kurt for facilitating my visit to the University of Utah, and allowing me use of his laboratory for a week. Thank you also to Ms. Megan O'Reilly, Dr. Foula Sozo and Professor Richard Harding (Department of Anatomy and Developmental Biology, Monash University) who developed the hyperoxic mouse model.

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PUBLICATIONS

MANUSCRIPTS

Sutherland MR, O'Reilly M, Ong K, Sozo F, Harding R, and Black MJ. Neonatal hyperoxia leads to glomerular hypertrophy in the adult mouse kidney [under revision, *AJP-Renal*] *(Chapter 5)*

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Gubhaju L, **Sutherland MR**, Yoder BA, Zulli A, Bertram JF, and Black MJ. Is nephrogenesis affected by preterm birth? Studies in a non-human primate model. *Am J Physiol Renal Physiol* 297: F1668-1677, 2009. *(My contribution to the research and preparation of the manuscript was conducted during the first year of my PhD)*

CONFERENCE ABSTRACTS

Developmental immaturity and postnatal injury: an evaluation of renal function in the preterm neonate **Megan R. Sutherland**, Lina Gubhaju, Alison M Medhurst, Rosemary SC Horne, Wendy Hoy, M. Jane Black. Europaediatrics Annual Congress, June 2011, Vienna, Austria *(oral presentation)*

Urinary NGAL: Is it predictive of pathological proteinuria in the preterm neonate? **Megan R. Sutherland**, Lina Gubhaju, Alison M Medhurst, Rosemary SC Horne, Wendy Hoy, M. Jane Black. Perinatal Society of Australia and New Zealand Annual Congress, April 2011, Hobart, Australia *(poster presentation)*

The effects of non-steroidal anti-inflammatory drugs and nitric oxide synthase inhibitors on the developing preterm kidney **Megan R. Sutherland**, Bradley A. Yoder, Ronald I. Clyman, M. Jane Black. Australian Health and Medical Research Congress, Nov 2010, Melbourne, Australia *(oral presentation)*

Immaturity or injury? An evaluation of renal function in the preterm neonate **Megan R. Sutherland**, Lina Gubhaju, Rosemary S. C. Horne, Alison M. Medhurst, M. J. Black. ASMR Student Research Symposium, June 2010, Melbourne, Australia (*poster presentation)*

The effect of preterm birth on nephrogenesis in the human kidney **Megan R. Sutherland**, Lina Gubhaju, Laura Stamp, Lynette Moore, Alison Kent, Jane E. Dahlstrom, M. J. Black. Perinatal Society of Australia and New Zealand Annual Congress April 2009, Darwin, Australia *(oral presentation)*

Preterm birth is associated with accelerated glomerular maturation and the formation of abnormal glomeruli **Megan R. Sutherland**, Lina Gubhaju, Laura Stamp, Lynette Moore, Alison Kent, Jane E. Dahlstrom, M. Jane Black. Australian & New Zealand Society of Nephrology Annual Scientific Meeting, Sept 2009, Hobart, Australia *(poster presentation)*

Renal effects in a mouse model of neonatal hyperoxia **Megan R. Sutherland**, Kimberly Ong, Megan O'Reilly, Foula Sozo, Richard Harding, M. Jane Black. Australian & New Zealand Society of Nephrology Annual Scientific Meeting, Sept 2009, Hobart, Australia *(poster presentation)*

Preterm birth is associated with accelerated renal maturation and abnormalities in glomerular morphology **Megan R. Sutherland**, Lina Gubhaju, Laura Stamp, Lynette Moore, Alison A. Kent, Jane E. Dahlstrom, M. Jane Black. 6th World Congress on the Developmental Origins of Health and Disease, Nov 2009, Santiago, Chile *(oral presentation)*

Evaluation of renal function in the preterm neonate: Evidence of microalbuminuria during the first month of life **Megan R. Sutherland**, Lina Gubhaju, Rosemary S. C. Horne, Alison M. Medhurst, M. Jane Black. 6th World Congress on the Developmental Origins of Health and Disease, Nov 2009, Santiago, Chile *(poster presentation)*

The effects of postnatal retinoic acid administration on nephrogenesis in the preterm baboon kidney **Megan R. Sutherland**, Lina Gubhaju, Mildred T. Stahlman, Bradley A. Yoder, M. Jane Black. Australian & New Zealand Society of Nephrology Annual Scientific Meeting, Sept 2008, Newcastle, Australia *(poster presentation)*

Retinoic acid administration following preterm birth in a non-human primate model: Effects on nephron endowment **Megan R. Sutherland**, Lina Gubhaju, Mildred T. Stahlman, Bradley A. Yoder, M. Jane Black. High Blood Pressure Research Council of Australia Annual Scientific Meeting, Dec 2008, Melbourne, Australia *(poster presentation)*

The effects of postnatal retinoic acid administration on nephron endowment in the preterm baboon kidney **Megan R. Sutherland**, Mildred T. Stahlman, Bradley A. Yoder, Lina Gubhaju, Jaqueline J. Coalson, M. Jane Black. Healthy Start for a Healthy Life: The Wintour's Tale - Satellite Conference of the World Congress on the Developmental Origins of Health and Disease, Nov 2007, Melbourne, Australia *(poster presentation)*

ABBREVIATIONS

AAP – American Academy of Pediatrics

AGA – Appropriate for gestational age

AHA – American Heart Association

AKI – Acute kidney injury

ANCOVA – Analysis of co-variance

ANOVA – Analysis of variance

ATPase - Adenosine-5'-triphosphate enzyme

β2-M – β2-microglobulin

BSA – Body surface area

C_{Cr} – Creatinine clearance

CO_2 – Carbon dioxide

COX – Cyclooxygenase enzyme

COX-1 – Cyclooxygenase enzyme 1

COX-2 – Cyclooxygenase enzyme 2

Cr – Creatinine

DCT – Distal convoluted tubule

DNA – Deoxyribonucleic acid

ENaC – Epithelial sodium channel

eNOS – Endothelial nitric oxide synthase

EUGR – Extrauterine growth restriction

ESKD – End stage kidney disease

ELISA - Enzyme-linked immunosorbent assay

FABP – Fatty acid binding protein

FE_{Na} – Fractional excretion of sodium

FSGS – Focal segmental glomerulosclerosis

GA – Gestational age

GBM – Glomerular basement membrane

GDNF – Glial cell-derived neurotrophic factor

GFR – Glomerular filtration rate

H^+ - Hydrogen ion

HMW – High molecular weight

IL-18 – Interleukin-18

IUGR – Intrauterine growth restriction

IVH – Intraventricular haemorrhage

K^+ - Potassium ion

KIM – Kidney injury molecule 1

LMW – Low molecular weight

L-NMMA – N^G-monomethyl-$_L$-arginine

MRI – Magnetic resonance imaging

Na^+ - Sodium ion

NAG – N-acetyl-beta-D-glucosaminidase

NHMRC – National Health and Medical Research Council of Australia

NKCC2 – Sodium, potassium, chloride co-transporter

NEC – Necrotising enterocolitis

NGAL – Neutrophil gelatinase associated lipocalin

NICU – Neonatal intensive care unit

NOS – Nitric oxide synthase

NOSi – Nitric oxide synthase inhibitor

NSAID – Non-steroidal anti-inflammatory drug

O_2 – Oxygen

PA – Postnatal age

PCT – Proximal convoluted tubule

PDA – Patent ductus arteriosus

PPROM – Premature pre-labour rupture of membranes

Rifle – Risk, injury, failure, loss, end-stage kidney disease

RAS – Renin angiotensin system

RC – Renal corpuscle

SD – Standard deviation

SEM – Standard error of the mean

SGA – Small for gestational age

SNGFR – Single nephron glomerular filtration rate

SpO2 – Blood oxygen saturation

TAL – Thick ascending limb of the loop of Henle

tAL – Thin ascending limb of the loop of Henle

tDL – Thin descending limb of the loop of Henle

TGF-β1 – Transforming growth factor- beta 1

USA – United States of America

UTP – Urine total protein

CHAPTER ONE

LITERATURE REVIEW

PRETERM BIRTH

1.1 EPIDEMIOLOGY OF PRETERM BIRTH

1.1.1 DEFINITION

Preterm birth is defined as birth prior to 37 completed weeks, or 259 days, of gestation (WHO, 1992). As shown in Figure 1.1, preterm birth can be further subdivided according to severity (Goldenberg *et al.*, 2008): near term (34-37 weeks gestation), moderately preterm (32-33 weeks gestation), very preterm (28-31 weeks gestation), and extremely preterm (less than 28 weeks gestation). Twenty-three weeks of gestation is considered to be the limit of viability for a preterm neonate, with the recommended clinical practice for infants born prior to this age, and those at less than 400 g body weight, being the provision of palliative care only (AHA and AAP, 2006).

Gestational age is conventionally determined by the timing of the mother's last menstrual period (WHO, 1992). For the most accurate assessment of gestational age, ultrasound measurements of fetal growth during pregnancy, as well as assessments of the physical and neurological maturity of the neonate after birth may also be made, such as through use of the Dubowitz clinical assessment criteria (Dubowitz *et al.*, 1970).

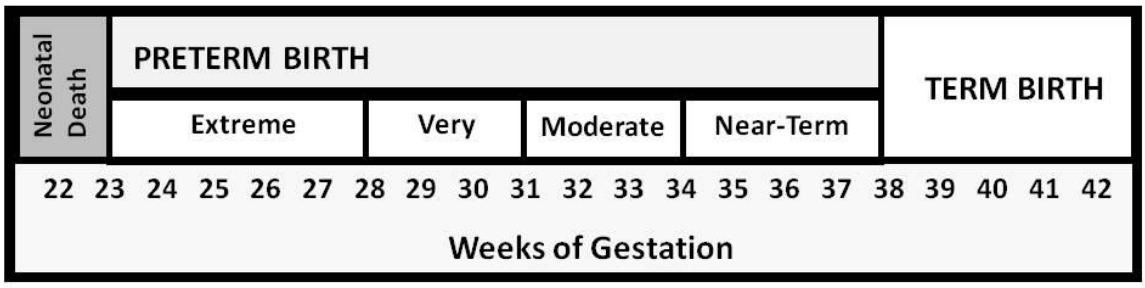

Figure 1.1: Preterm birth is defined as birth prior to 37 completed weeks of gestation, and is further subdivided according to severity: 23 weeks is considered the limit of viability for a preterm neonate, with neonatal/fetal death occurring before this time; 23-27 weeks extremely preterm; 28-31 weeks very preterm; 32-33 weeks moderately preterm; 34-37 weeks near term. Term infants are born after 37 completed weeks of gestation.

1.1.2 INCIDENCE

Preterm birth currently accounts for 8.2% of all births in Australia (Laws *et al.*, 2010), and 12.3% in the USA (Mathews *et al.*, 2011). Worldwide, the rates of preterm birth average 9.6% which equates to 12.9 million births annually (Beck *et al.*, 2010). In developing countries, however, the incidence of preterm birth is generally higher; for example, the rate of preterm birth in southern Africa is 17.5% (Beck *et al.*, 2010).

Within Australia, rates of preterm birth are highest within the Aboriginal and Torres Strait Islander population, with 13.3% of Indigenous babies born preterm compared to 8.0% in the non-Indigenous population (Laws *et al.*, 2010). Of all Australian states and territories in 2008, the highest rate of preterm birth (9.8%) was in the Northern Territory, with the lowest rate (7.5%) in New South Wales (Laws *et al.*, 2010). The majority (56.7%) of preterm births in Australia were of twins and higher order multiple births, whereas just 6.6% of singletons were preterm (Laws *et al.*, 2010).

In general, the proportion of preterm births is different for each gestational age category, with the majority of preterm births being near-term; approximately 60-70% of preterm births are at 34-36 weeks gestation, 20% at 32-33 weeks, 15% at 28-31 weeks, and just 5% of preterm births are at less than 28 weeks gestation (Goldenberg *et al.*, 2008). In Australia, 6.5% of the total births in 2008 were at 32–36 weeks gestation, 0.8% were at 28–31 weeks gestation, and 0.9% at 20–27 weeks gestation (Laws *et al.*, 2010), demonstrating that there was a very similar percentage of babies born extremely and very preterm. It needs to be taken into account, however, that the babies born prior to 23 weeks gestation would not have survived.

Importantly, the incidence of preterm birth continues to rise worldwide (Martin *et al.*, 2006; Shennan and Bewley, 2006; Tracy *et al.*, 2007). The rates of preterm birth in Australia have risen from 6.4% in 1990 to 8.2% in 2008 (Roberts and Lancaster, 1999; Laws *et al.*, 2010), and similarly in the USA rates have increased from 10.6% to 12.3% over the same time period (Heron *et al.*, 2010; Mathews *et al.*, 2011). This increase in rates is largely attributed to the escalating use of assisted reproductive therapies, which has increased the number of multiple births, and in addition, the rise in the number of deliveries following clinical intervention (Slattery and Morrison, 2002; Goldenberg *et al.*,

2008). Shennan and Bewley (2006) have also suggested that more accurate assessments of gestational age in recent times may also be contributing to the rising percentage of neonates classified as being preterm.

1.1.3 CAUSES OF PRETERM DELIVERY

Preterm birth may occur due to the spontaneous onset of preterm labour, premature pre-labour rupture of membranes (PPROM), or may be clinically indicated due to maternal and/or fetal health risks; in each of these cases, the birth of the infant may be by vaginal or caesarean delivery (Goldenberg *et al.*, 2008). The majority of preterm births (approximately 40%) occur due to spontaneous preterm labour, 25% occur following PPROM, and 35% are clinically indicated (Goldenberg *et al.*, 2008).

The cause of preterm birth is often multifactorial in origin. As reviewed by Goldenberg *et al*. (2008), a large number of risk factors for spontaneous premature labour and PPROM have been identified. These include: maternal race (highest risk in black and Indigenous women, lowest risk in Hispanic and east Asian women), previous preterm delivery, a short time interval between pregnancies, low maternal body mass index, maternal smoking, multiple pregnancy, maternal stress, and also maternal medical conditions such as depression, cervical incompetence, thyroid disease, asthma, diabetes, and hypertension. The most significant contributor to preterm birth is intrauterine infection (including chorioamnionitis), which may be involved in up to 40% of preterm births (Goldenberg *et al.*, 2000). Clinical indications for preterm delivery include risk factors for maternal and fetal health such as pre-eclampsia, placental abruption, placenta praevia, chorioamnionitis, intrauterine growth restriction (IUGR) and abnormal amniotic fluid levels (oligohydramnios or polyhydramnios); in these cases labour will be medically induced, or an emergency caesarean will be performed.

1.1.4 MORBIDITY AND MORTALITY

SURVIVAL RATES

Approximately 75% of perinatal deaths occur in neonates that were delivered preterm (Slattery and Morrison, 2002). Rates of survival are strongly associated with gestational

age at birth, with the most immature neonates having the highest risk of perinatal mortality. Infants born very preterm (28-31 weeks gestation) have survival rates at approximately 80%, whereas moderately preterm and near-term infants (born after 32 weeks gestation) have survival rates similar to term infants, at near to 100% (Slattery and Morrison, 2002). In neonates born extremely preterm (less than 28 weeks gestation), there is a significant drop in survival rates with every one week reduction in gestational age at birth; survival rates for neonates born at 23 weeks gestation are approximately 20%, whereas 60% of neonates at 26 weeks gestation survive the perinatal period (Slattery and Morrison, 2002).

Survival rates have significantly increased over the last two decades due to technological advancements and improvement in clinical care practices, in particular due to the administration of surfactant and antenatal glucocorticoids which aid lung maturation and function (Roberts and Dalziel, 2006; Stevens *et al.*, 2007). There is still much variation, however, in mortality rates between different countries, and also between hospitals within the same regions depending on their level of neonatal care (Saigal and Doyle, 2008). In studies conducted by the Victorian Infant Collaborative Study Group (Australia), the survival of infants born extremely preterm at 24 weeks gestation increased from 12% in 1985-1987 to 33% in 1991-1992, and further increased to 41% by 1997 (Doyle, 2004; Yu and Doyle, 2004). Importantly, survival has also recently become possible for those neonates born at earlier gestational ages. In those neonates born as young as 23 weeks gestation, there were no survivors in 1985-87, however 10% survived in 1991-1992, and 41% survived in 1997 (Doyle, 2004; Yu and Doyle, 2004). The most common causes of death in preterm neonates are related to infection, respiratory disease and cardiovascular disease (Barton *et al.*, 1999; Elder and Zuccollo, 2005; Kitsantas, 2008).

COMMON MORBIDITIES

Although the mortality rates of preterm neonates have significantly decreased over time, there has been little change (or even an increase) in the percentage of neonates who suffer either neonatal (de Kleine *et al.*, 2007; Fanaroff *et al.*, 2007) or long-term (Yu and Doyle, 2004) disability or disease. Compared to term-born infants, preterm neonates have significantly higher rates of illness and disease in the neonatal period; common

conditions include respiratory distress syndrome, early and late-onset sepsis, intraventricular haemorrhage, patent ductus arteriosus, and necrotising enterocolitis (Fanaroff *et al.*, 2007).

In the long-term, survivors of preterm birth suffer a multitude of both physical and neurological problems, which include: sensory loss (deficits in sight or hearing), cerebral palsy, cognitive deficits and behavioural problems (learning difficulties), respiratory disease, impaired growth, and functional disabilities (Hack, 2006; Moster *et al.*, 2008; Saigal and Doyle, 2008). The risk of long-term disease is strongly correlated with gestational age at birth; in a cohort of 903,402 subjects born in Norway between 1967-1983 and assessed in 2003 (aged 20-36 years), 10.6% of those born at less than 28 weeks gestation, 8.2% born at 28-30 weeks, 4.2% at 31-33 weeks, and 2.4% at 34-36 weeks gestation were classified as having a medical disability that severely affected working capacity (Moster *et al.*, 2008).

1.2 PHYSIOLOGY OF PRETERM BIRTH

1.2.1 HAEMODYNAMIC CHANGES AT BIRTH

At the time of birth, and in the early postnatal period, major transitional events occur as the neonate adjusts to extrauterine life. This transition involves significant changes in both the structure and function of the fetal vasculature and organs.

CARDIOVASCULAR CIRCULATION

The most prominent of these changes is the transition from a parallel (fetal) to an in-series (adult) cardiovascular circulation. The clamping of the umbilical cord after birth firstly eliminates all blood flow from the placental circulation and necessitates the neonate to function independently. Newborn neonates initiate their first breath within an average of 18 seconds after delivery (Saunders and Milner, 1978), or in the case of the preterm infant with immature lung function, resuscitation and ventilation also commence soon after delivery (AHA and AAP, 2006); the subsequent clearance of lung liquid and increased vascular surface area for gas exchange (through vasodilator activity and

structural alterations of pulmonary vessels) leads to an 80% drop in pulmonary vascular resistance (Blackburn, 2007; Gao and Raj, 2010). Correspondingly, pulmonary blood flow rapidly increases from 138 ml/min/kg in the fetus to 245 ml/min/kg in the newborn (Gao and Raj, 2010). The increased blood return through the pulmonary vein increases pressure in the left ventricle of the heart, which becomes the dominant ventricle after birth (Blackburn, 2007). Increased left ventricular load is associated with structural and functional changes in the ventricular wall, with increased cardiomyocyte length, increased myofibril production, and an improved ability to generate force (Blackburn, 2007). Recent experimental studies in an ovine model have shown further remodeling of the myocardium following preterm birth, with altered cardiomyocyte maturation, stress-related changes in the DNA, and increased collagen deposition in the heart (Bensley *et al.*, 2010). Compositional changes to the wall of the aorta and pulmonary artery, as well as vascular injury have also been observed following preterm birth in the sheep model (Bensley *et al.*, 2012).

BLOOD PRESSURE AND DISTRIBUTION OF BLOOD FLOW

Changes in blood pressure and the redistribution of blood flow after birth, as well as the activity of vasodilatory mediators and increased blood oxygen concentrations all contribute to the closure of three vascular shunts. These shunts functioned in the fetus to enable oxygenated blood to reach the heart directly, and for the majority of blood flow to bypass the lungs *in utero* (Kiserud, 2005; Blackburn, 2007; Gao and Raj, 2010); namely, the foramen ovale (opening in the septum of the heart, connecting the left and right atrium), the ductus arteriosus (fetal vessel connecting the pulmonary artery and the aortic arch) and the ductus venosus (fetal vessel connecting the umbilical vein and the inferior vena cava). Functional closure of the ductus arteriosus occurs in the majority of term neonates within 72 hours of birth, followed by anatomical closure which may take several weeks (Clyman, 2006; Blackburn, 2007). In the preterm neonate, however, prolonged patency of the ductus arteriosus occurs in 65% of neonates born at less than 30 weeks gestation (Clyman, 2006). Treatment of a clinically significant patent ductus arteriosus involves either surgical ligation of the duct, or most commonly, pharmacological treatment with non-steroidal anti-inflammatory drugs (NSAIDs), either indomethacin or ibuprofen.

Importantly, there is a large increase in systemic arterial blood pressure after birth, rising from 30-40 mmHg at 28 weeks gestation *in utero* (Kiserud, 2005) up to approximately 75 mmHg postnatally (Rudolph, 1970). Blood flow redistribution is also evident after birth. The majority of fetal cardiac output (up to 50%) is directed to the placenta, 14% to the brain, 10-15% to the lungs, and less than 5% to the kidneys (Rudolph, 1970; Rudolph *et al.*, 1971; Blackburn, 2007). With the removal of the placental circulation and increased functional demand, however, blood flow to systemic organs in the newborn significantly increases; approximately 6% of cardiac output perfuses the kidneys by 12 hours after birth, increasing to 10% within the first two days (Blackburn, 2007).

BLOOD OXYGEN CONCENTRATION

Blood oxygen concentrations also change significantly in the transitional period after birth. The intrauterine environment is relatively hypoxic (Rodesch *et al.*, 1992; Fischer and Bavister, 1993), with blood oxygen saturation levels (SpO_2) in the fetal circulation significantly lower than in the maternal circulation, averaging 45-55% (Finer *et al.*, 2010). After term birth, SpO_2 in the neonate rises rapidly, reaching 80-90% within the first five minutes (Kamlin *et al.*, 2006; Rabi *et al.*, 2006). In preterm infants, supplemental oxygen therapy is commonly used in order to achieve sufficient blood oxygen saturation in immature lungs. However, the neonatal blood oxygen saturation levels that are most favourable for postnatal growth and development, and the concentration of oxygen optimal for initial resuscitation (ranging from 21% to 100% (Tan *et al.*, 2005; Rabi *et al.*, 2011)) or for the short to long-term care (Carlo *et al.*, 2010) of the preterm neonate remain undefined and thus vary significantly between individual practitioners and between institutions (Deuber and Terhaar, 2011).

Importantly, even very brief exposures to high oxygen concentrations are known to cause oxidative stress, leading to the production of oxygen free radicals which subsequently cause cellular injury and cell death (Vento *et al.*, 2001; Vento *et al.*, 2003). The preterm neonate is particularly vulnerable due to low concentrations of antioxidants at birth (Georgeson *et al.*, 2002; Lee and Chou, 2005); common diseases of prematurity such as bronchopulmonary dysplasia, retinopathy of prematurity, necrotising enterocolitis,

patent ductus arteriosus and periventricular leukomalacia are all strongly linked to oxidative stress in the preterm neonate (Saugstad, 2001).

1.2.2 POTENTIAL CONSEQUENCES FOR THE PRETERM KIDNEY

Preterm neonates are born at a time when organ development is still ongoing; therefore, they are prematurely and abruptly exposed to the extrauterine environment in which the organs of the preterm infants are often not structurally mature enough to function adequately. The extrauterine environment differs significantly from the *in utero* environment which is optimal for fetal organogenesis. In particular, blood oxygen concentration and systemic blood pressure are increased, the environmental temperature is colder, nutritional needs are not constantly supplied by the maternal circulation, exposure to medications is common, and the neonatal organs must function independently and at a higher level of demand. Therefore, it is likely that any ongoing organ development in the preterm neonate after birth may be impaired.

The kidney is one organ in which development is normally ongoing late in gestation (Saxen, 1987; Hinchliffe *et al.*, 1991), and therefore is likely to be adversely affected by preterm birth. Fetal fluid homeostasis is controlled by the maternal kidney via the placenta (Beall *et al.*, 2008). After birth, however, increased demand is placed on the neonatal kidney as it must function independently. Postnatally, factors associated with the haemodynamic transition to extrauterine life (such as changes in blood pressure and flow distribution, and blood oxygen concentrations) as well as factors associated with the postnatal clinical care of the preterm neonate (including supplemental oxygen therapy and exposure to nephrotoxic medications) may influence renal development and function in the early postnatal period. Given the increasing numbers of preterm neonates being born each year worldwide (Martin *et al.*, 2006; Shennan and Bewley, 2006; Tracy *et al.*, 2007), and the potential for long-term renal disease following impaired renal development (Hoy *et al.*, 2005), it is essential to determine the effects of preterm birth on renal development and function. Furthermore, it is important to delineate the effects of individual haemodynamic/clinical factors in order to work towards optimising the renal health of neonates born preterm.

RENAL DEVELOPMENT AND MATURATION

2.1 RENAL ANATOMY AND FUNCTION

The fully formed human renal system consists of paired kidneys which are situated retroperitoneally on the posterior abdominal wall and inferior to the diaphragm at the level of the 12th thoracic to the 3rd lumbar vertebrae. Each kidney is enclosed by a fibrous capsule, beneath which the parenchyma of the kidney is divided into an outer region of cortex and an inner region of medulla. As shown in Figure 2.1, the parenchyma is also divided laterally into 8-18 conical lobes, named the renal pyramids (Kerr, 1999). The base of each lobe, the papilla, is the region of urine flow from the collecting duct into the minor calyces through a porous cribiform plate (Hallgrimsson *et al.*, 2003). The minor calyces subsequently merge into two or three major calyces, forming the renal pelvis (Kerr, 1999); the medial region of the renal pelvis narrows and becomes continuous with the ureter.

2.1.1 THE NEPHRON

The kidney serves as a regulator of fluid homeostasis, acid-base and electrolyte balance, arterial blood pressure, metabolic waste excretion, and is also involved in hormone production. All of these functions occur through the activity of the nephron, the fundamental functional unit of the kidney. In the human kidney, nephron number can range from approximately 200,000 to over two and a half million (Puelles *et al.*, 2011a).

As shown in Figure 2.1, the major structural components of the nephron are the renal corpuscle and associated renal tubules. The renal corpuscle is composed of the glomerulus (tuft of glomerular capillaries), surrounded by the Bowman's capsule; blood flowing through the glomerular capillaries is filtered by a glomerular filtration barrier (Patrakka and Tryggvason, 2010), and the resultant filtrate passes through the Bowman's space and into the proximal tubule. Passage of the filtrate through each distinct region of the renal tubule (Figure 2.1) ultimately results in the reabsorption of essential molecules such as sodium (Chevalier, 2001) and protein (Christensen and Birn, 2001), and the dilution/concentration of the urine (Sands and Layton, 2009).

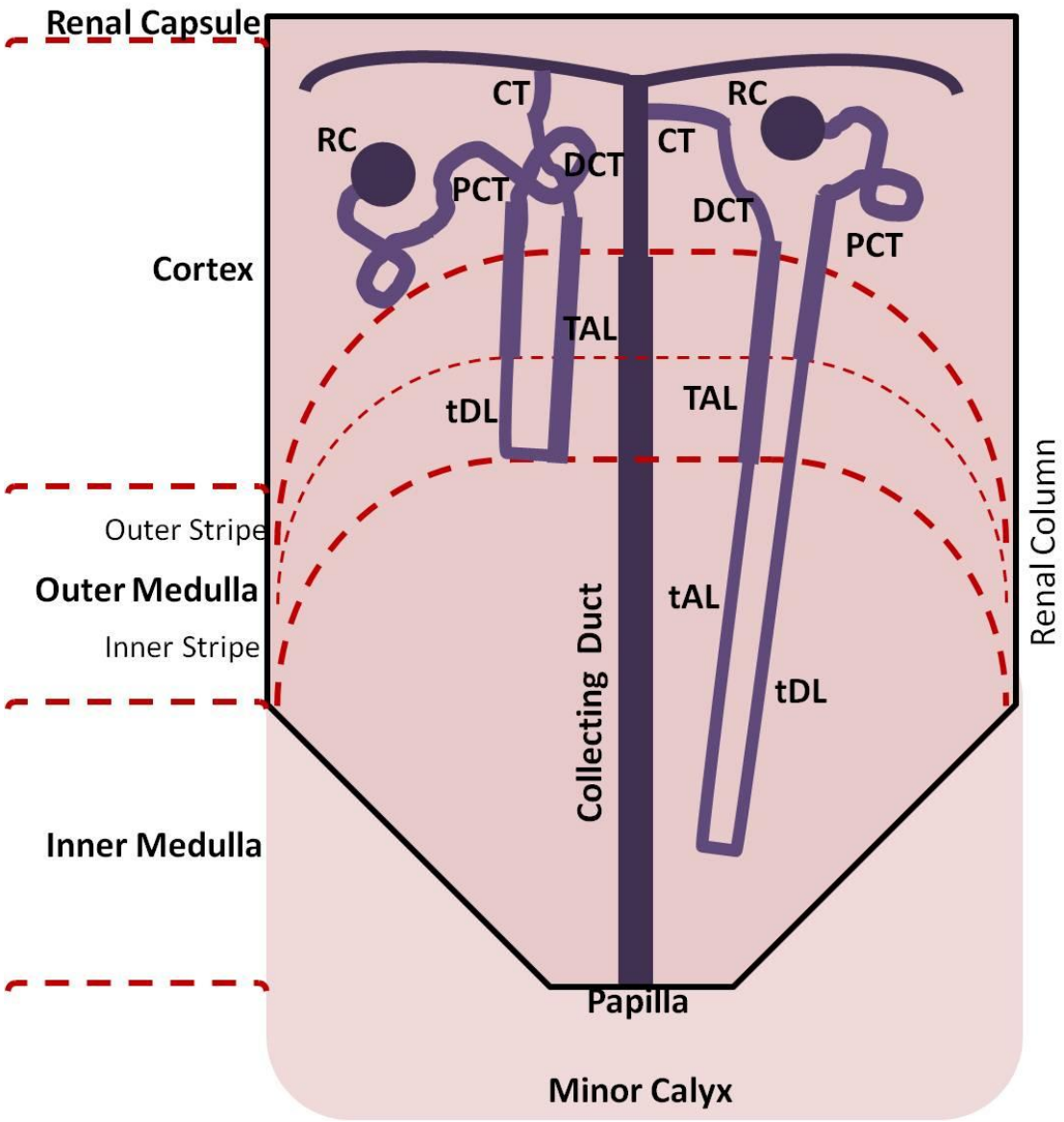

Figure 2.1: Graphic illustration of the structure of renal pyramids, based on Kerr (1999). Each pyramid is capped by renal cortex in the outer kidney, in which all renal corpuscles are located. The cortex extends downwards on each side to form the lateral borders known as the renal columns. Beneath the cortex is the outer stripe of the outer medulla (containing only thick ascending and descending tubules) and the inner stripe of the outer medulla (containing the loop of Henle of short-looped nephrons (shown on left)). Long-looped nephrons (shown on right) extend into the inner medulla. At the base of the pyramid, urine flows from the collecting duct through the porous cribiform plate of the papilla into the minor calyx. Segments of the nephron include the renal corpuscle (**RC**), the proximal convoluted tubule (**PCT**), thin descending limb of the loop of Henle (**tDL**), thin ascending limb of the loop of Henle (**tAL**), thick ascending limb of the loop of Henle (**TAL**), distal convoluted tubule (**DCT**), and the connecting tubule (**CT**) which is continuous with the collecting duct.

2.2 RENAL DEVELOPMENT

The human kidney develops through three successive and overlapping phases, beginning at 22 days of gestation (Saxen, 1987). Initially, transient and largely non-functional structures known as the pronephros and mesonephros form, from which the mature kidney (metanephros) arises. The pronephros and mesonephros stages of development are completed by 9 weeks gestation, with regression of the rudimentary tubules occurring from the 5th week. During this time the nephric duct, a pivotal component of the developing excretory system, is formed (Dressler, 2006). From the nephric duct all tubules originate, extending medially into a region known as the nephrogenic cord (Dressler, 2006).

2.2.1 METANEPHROS

Two distinct tissue types contribute to the formation of the metanephric kidney, with cells from both the mesenchymal nephric cord and the mesoderm-derived nephric duct involved (Saxen, 1987). Formation of the permanent kidney begins in the 5th week of gestation (Osathanondh and Potter, 1963) with an outgrowth of cells from the caudal end of the nephric duct termed the ureteric bud. Reciprocal interactions subsequently occur between the ureteric bud and the metanephric mesenchyme, a dense mass of embryonic cells located at the base of the nephric cord. These interactions, primarily involving the Ret receptor (expressed on the tips of the ureteric bud) and its mesenchyme-derived ligand glial cell line-derived neurotrophic factor (GDNF) (Davies, 2003; Costantini and Shakya, 2006), result in the differentiation of the mesenchyme to form nephrons, and the growth and bifurcation of the ureteric bud to form the collecting ducts (Saxen, 1987). For an up to date description on the molecular control of branching morphogenesis and nephrogenesis, see Faa *et al*. (2012).

2.2.2 BRANCHING MORPHOGENESIS

The architecture of nephron arrangement within the human kidney is derived through the process of branching morphogenesis (Figure 2.2); the efficiency of branching is regarded as an important determinate of final nephron endowment within the kidney. As

described in the work of Osathanondh and Potter (1963), the development of the kidney occurs through four distinct periods:

Period 1: The first period of branching morphogenesis begins at week 5 of gestation and continues until the 14^{th}-15^{th} week. The first branching of the ureteric bud is symmetrical and occurs at an angle of approximately 180°. The subsequent generations of branching occur at increasingly narrower angles, and may be either symmetrical or asymmetrical. The first 5 generations of branches later dilate to form the renal pelvis and major and minor calyces. The following generation forms the papillary ducts, with the remainder forming individual collecting ducts.

After the ureteric bud has branched for 3-5 generations, nephrons are induced to differentiate at the tips of the latest branches. As the ampullae continue to divide, the point of attachment of nephrons that have already begun to form advance along with the bud tip, and the nephrons are carried forward to successive generations (Figure 2.2A). Overall, 6-8 generations of branches form distal to the minor calyces, and by the completion of period 1, ureteric bud branching is generally completed.

Period 2: Period 2 of development occurs from week 14-15 of gestation and continues until 20-22 weeks. During this period, the ampullae no longer actively divide and can induce further nephrons to form at branch tips that have already generated a nephron during period 1. When the second nephron for the branch point is formed, both are temporarily attached via their collecting tubules to the same ampullae. The point of attachment of the first nephron then shifts away from the ampullae onto the collecting tubule of the newly formed second nephron. This process of nephron attachment to a single collecting duct continues until 2-8 nephrons are formed in the arcade arrangement (Figure 2.2B).

Period 3: Period 3 lasts from 20-22 weeks of gestation until the completion of nephrogenesis at 32-36 weeks of gestation. During this period, the ampullae advance beyond the point of attachment of the nephrons in the arcade. New nephrons that are formed at the ampullae attach individually to the terminal end of the ureteric branch. A further 4-7 generations of nephrons are formed during this period (Figure 2.2C). By the completion of period 3, the oldest (most mature) nephron is positioned near to the

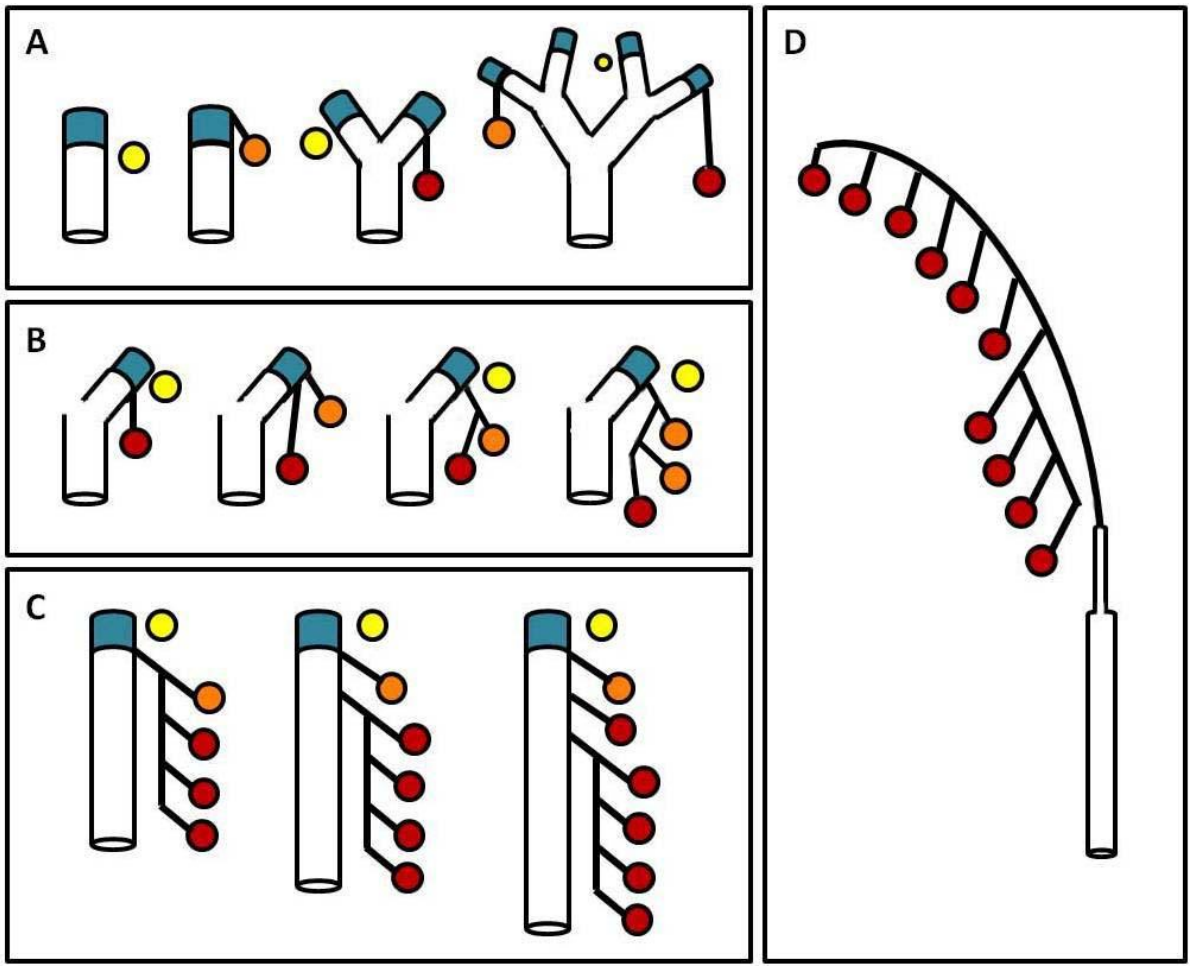

Figure 2.2: Graphic illustration of the stages of branching morphogenesis, based on Osathanondh & Potter (1963). Period one (**A**) of branching morphogenesis involves the differentiation of nephrons, and the branching of ampullae. Each nephron that is formed is carried from its point of attachment to successive generations of branches. In period two (**B**) nephrons are induced at the tips of branches in which a nephron has already formed; the points of attachment of the nephrons shift from the ampullae to the collecting tubule of the previously formed nephron, in an arcade pattern. Nephrons formed in period three (**C**) attach individually to the terminal end of the ureteric branch. The final architecture of the mature kidney involves up to 8-10 generations of nephrons (**D**), including 4-5 attached in arcades (periods one and two; inner cortex) and the remainder singly attached to the collecting duct (period three; outer cortex). Ampullae are shown in blue, collecting tubules in white, vesicles in yellow, S-shaped bodies in orange, and mature nephrons in red.

medulla, and has the longest loop of Henle. The most recently formed period 3 nephrons are positioned in the outer renal cortex beneath the renal capsule (Figure 2.2D).

Period 4: Period 4 begins when the final ampullae disappear at approximately 32-36 weeks gestation. During this period no new branches or nephrons are formed, and the period is characterised by interstitial growth and differentiation. By the completion of this period of development each terminal branch has approximately 10 generations of nephrons attached to it (Figure 2.2D). No new nephrons are formed following the completion of nephrogenesis, for the lifetime of the individual.

2.2.3 NEPHROGENESIS

Reciprocal signaling between the ureteric bud epithelium and the cells of the surrounding metanephric mesenchyme induces the formation of nephrons at the ampullae (Davies, 2003). Firstly, the mesenchymal cells condense into an aggregate adjacent to the tip of the ureteric bud. Following this, the aggregated cells undergo a mesenchyme-to-epithelial cell conversion, rapidly proliferating before forming a renal vesicle (Dressler, 2006). The vesicle then elongates and a cleft is formed in the proximal region to form a comma-shaped body. A further cleft is subsequently generated in the distal region of the comma-shaped body, forming the S-shaped body (Osathanondh and Potter, 1963; Saxen, 1987; Dressler, 2006).

The S-shaped body is patterned along the proximal-distal axis; the upper (distal) limb fuses with the ureteric bud to form a continuous tubule, the cells of which differentiate into cuboidal epithelium and elongate and twist to form the distal convoluted tubule (Dressler, 2006). The central region of the S-shaped body elongates and loops to form the loop of Henle, followed by the proximal convoluted tubule; the lower (proximal) limb of the S-shaped body develops into the renal corpuscle (Osathanondh and Potter, 1963; Saxen, 1987; Dressler, 2006).

2.2.4 GLOMERULOGENESIS

The renal corpuscle arises from the lower limb of the S-shape body through a process of glomerulogenesis. Firstly, the inner cells of the lower cleft of the S-shape body

differentiate into the glomerular (visceral) podocytes, while the outer cells form the parietal podocytes which line the Bowman's capsule (Osathanondh and Potter, 1963; Saxen, 1987; Dressler, 2006). During the differentiation of podocytes, endothelial precursor cells from the mesenchyme invade the lower cleft, and differentiate to form the capillary loop (Abrahamson and Wang, 2003). During this time both the podocyte epithelium and the developing endothelial cells actively synthesise the components required to form the shared glomerular basement membrane (GBM). The podocytes fuse with the developing GBM and further differentiate to form foot processes; the endothelium flattens and becomes fenestrated (Abrahamson and Wang, 2003).

GLOMERULAR CAPILLARISATION

Current experimental evidence indicates that the metanephric mesenchyme is the source of endothelial progenitor cells, the angioblasts, which begin to divide within the lower cleft of the developing glomerular S-shaped body (Hyink *et al.*, 1996; Abrahamson and Wang, 2003); the development of the glomerular capillaries therefore occurs through *in situ* vasculogenesis (Robert *et al.*, 1998; Woolf and Loughna, 1998; Ballermann, 2005).

Development of the glomerular vasculature is initiated following the secretion of growth factors from the differentiating podocytes, with vascular endothelial growth factor (VEGF) being the key growth factor responsible for the development and maintenance of the glomerular capillaries (Eremina and Quaggin, 2004). Within the cleft of the developing glomerulus, the angioblasts undergo homotypic aggregation and form into pre-capillary cords (Ballermann, 2005). The lumen of the cords is formed through transforming growth factor β1 (TGF-β1)-dependent apoptosis of a subset of endothelial cells (Fierlbeck *et al.*, 2003). The final stage of glomerular capillary formation involves the flattening of the remaining endothelial cells along the glomerular basement membrane, and the formation of the extensive fenestrae (Ballermann, 2005); VEGF has been demonstrated to be critical to this process (Roberts and Palade, 1995; Eremina and Quaggin, 2004). The glomerular capillaries are continuous with the afferent and efferent arterioles which enter/exit the renal corpuscle at the vascular pole. The origin of the arterioles and larger renal vessels, however, is largely unknown (Woolf and Loughna, 1998).

2.2.5 FUNCTIONAL DEVELOPMENT

In utero, the fetal kidney does exhibit some function despite control of fluid homeostasis being provided by the maternal kidneys via the placenta. Fetal urine constitutes an important component of the amniotic fluid, which is essential for fetal growth and development (Moritz and Wintour, 1999). Progressive developmental changes in the kidney involve modification of renal blood flow, increased glomerular filtration rate (GFR; rate of flow of filtrate through the glomerular filtration barrier), and alterations in renal sodium handling.

RENAL BLOOD FLOW

Blood flow to the kidneys is very low during fetal life, with a fetus at 10-20 weeks gestation only receiving 5% of cardiac output (Rudolph *et al.*, 1971). Renal blood flow subsequently increases to approximately 9% following term birth, and 25% by adulthood; this is a high proportion of blood flow considering the combined adult kidneys constitute just 0.5% of total body mass (Rudolph *et al.*, 1971; Satlin *et al.*, 2003).

The increase in renal blood flow throughout the period of renal development is due to increases in mean arterial pressure, cardiac output and primarily to a decrease in renal vascular resistance (Gruskin *et al.*, 1970; Jose *et al.*, 1971; Satlin *et al.*, 2003). Resistance of the afferent and efferent arterioles is a determinant of glomerular capillary pressure, and thus influences GFR. The significant decrease in renal vascular resistance is likely due to changes in the balance of vasoconstriction via the renin-angiotensin system (RAS activity is inversely related to gestational and postnatal age (Smith *et al.*, 1974)), and vasodilatory factors such as nitric oxide (Solhaug *et al.*, 1996). Furthermore, structural changes in the developing kidney, such as the formation of new vasculature and glomeruli, are also likely to reduce overall resistance.

Besides an increase in total renal blood flow during development, there are also changes in the distribution of blood flow within the kidney, as has been demonstrated through experimental studies using various animal models. During the last trimester of gestation, the perfusion rate for nephrons within all regions of the cortex remains stable (Robillard *et al.*, 1981). In the newborn, however, intrarenal blood flow is distributed primarily to

the inner cortex and medulla. By six weeks of age, there is a significant increase in blood flow to the outer renal cortex (Robillard *et al.*, 1981), likely related to increased endothelial nitric oxide synthase (eNOS) levels (Ratliff *et al.*, 2007); this pattern of blood distribution continues into adulthood (Olbing *et al.*, 1973).

Overall, the haemodynamic adaptations of the fetal kidney to extrauterine life involves transformation from a high vascular resistance fetal organ with low blood flow, into a high blood flow, low vascular resistance organ, with primary blood supply to the renal cortex (Satlin *et al.*, 2003). The low blood flow to the outer renal cortex, as seen in animal models at the time of term birth, is likely due to the presence of developing, immature glomeruli in this region of the kidney. It is unknown, however, whether this protective pattern of blood distribution is also present in the case of the preterm human neonate, where nephrogenesis is also ongoing following birth.

GLOMERULAR FILTRATION RATE

Assessments of glomerular function are achieved through the measurement of GFR. GFR refers to the rate of flow of a either an exogenous (inulin) or endogenous (creatinine) filtration marker through the glomerulus, measured in ml/min and traditionally corrected for by body surface area (averaging 1.73 m^2 in adults). Serum creatinine levels, and the calculation of creatinine clearance (C_{Cr}; which takes into account both serum and urine creatinine levels) are common proxy measures of GFR for use in the clinical setting (Stevens *et al.*, 2006).

Glomerular filtration in the fetus begins after the first formation of nephrons at approximately 9 weeks of gestation (Satlin *et al.*, 2003). Measurements of GFR *in utero* show that it remains low, even when corrected for fetal body weight (Robillard *et al.*, 1975), but is strongly correlated with gestational age (Satlin *et al.*, 2003). There is a non-linear increase in GFR over the period of gestation, with a more rapid increase occurring after approximately 34 weeks gestation (Arant, 1978; Satlin *et al.*, 2003); this coincides with the timing of the completion of nephrogenesis.

The pattern of increases in single nephron glomerular filtration rate (SNGFR) during nephrogenesis follows the centrifugal pattern of glomerular perfusion (Satlin *et al.*, 2003);

the most mature nephrons have higher filtration rates than do the newly formed nephrons in the outer renal cortex (Aperia *et al.*, 1977). Increases in total kidney GFR throughout development are therefore primarily attributable to increased perfusion of nephrons, following changes in total renal blood flow and its distribution, and a drop in renal vascular resistance (Aperia *et al.*, 1974; Robillard *et al.*, 1981). Furthermore, ongoing structural growth and maturation of nephrons leads to an increase in glomerular filtration surface area, and some increases in the permeability of the glomerular filtration barrier have also been reported (Goldsmith *et al.*, 1986; Satlin *et al.*, 2003).

Immediately following birth, serum creatinine levels are equivalent to the fetal levels, which during the third trimester of gestation rise from 42 µmol/L at 23 weeks to 47 µmol/L at term; the increase likely reflecting an increase in muscle mass (Moniz *et al.*, 1985). In the first forty-eight hours following birth, however, serum creatinine levels significantly increase (Bueva and Guignard, 1994; Miall *et al.*, 1999). This is considered to be due in part to tubular creatinine reabsorption, as has been evidenced in a neonatal animal model (Matos *et al.*, 1998), and also due to the inadequacy of glomerular filtration during the early postnatal period (Miall *et al.*, 1999). Peak serum creatinine levels are reached between postnatal days 2-4 of life, with the highest levels and most delayed timing of the peak creatinine level seen in neonates at the lowest gestational ages (Miall *et al.*, 1999).

GFR continues to increase postnatally; by one year of age, 90% of adult GFR levels have been reached. GFR levels of 130 ml/min/1.73m^2 in males and 120 ml/min/1.73m^2 in females are considered to be normal for young adults (Stevens *et al.*, 2006; Rhodin *et al.*, 2009).

SODIUM EXCRETION

An important measure of tubular function is the calculation of the fractional excretion of sodium (FE_{Na}), the percentage of sodium that is excreted and not taken up through tubular reabsorption. The calculation of FE_{Na} takes into account both plasma and urine sodium, and is corrected by plasma and urine creatinine levels. The primary rationale for examining the fractional excretion of sodium is to analyse the function of the Na^+

transporters in the proximal tubule, as these are responsible for the majority of sodium reabsorption within the nephron (Corman and Roinel, 1991).

The FE_{Na} is very high in the fetus, decreasing from over 5% at 28 weeks gestation, to below 0.2% in the term newborn (Siegel and Oh, 1976); the term neonate therefore maintains a positive sodium balance (Engle, 1986) which is continued into adulthood. Unlike the adult kidney, however, the high retention of sodium in the neonate renders them with a limited capacity to excrete a sodium load, which may result in hypernatraemia and oedema (Dean and McCance, 1949; Aperia *et al.*, 1972; Satlin *et al.*, 2003). The inadequate increase in the FE_{Na} following a sodium load in the neonate is independent of GFR; therefore, it is likely due to blunted diuretic and naturetic responses to a sodium load, or differences in the structure of the renal tubule (Satlin *et al.*, 2003).

In experimental studies using animal models, differences in the renal handling of sodium have been shown to occur with increasing maturation. In the young animal, there is reduced efficiency of sodium reabsorption in the proximal tubule and loop of Henle, with 35% of filtered sodium reaching the distal tubule (Lelievre-Pegorier *et al.*, 1983); in the adult kidney, just 5-10% of the filtered sodium passes through to the distal tubule (Malnic *et al.*, 1966; Satlin *et al.*, 2003). Within the distal convoluted tubule, however, the reabsorption of sodium is much greater in younger animals, which may contribute to their high sodium retention (Aperia and Elinder, 1981; Satlin *et al.*, 2003).

Underlying the maturational changes in the FE_{Na} is likely the molecular upregulation of Na^+ transport proteins that occurs after birth, and changes in tubular cell structure. With increasing maturation of the proximal tubule, there is a doubling of the basolateral surface area (Evan *et al.*, 1983; Aperia and Larsson, 1984), and an increase in the abundance and activity of Na^+/K^+/ATPase transporters (Aperia *et al.*, 1981b; Guillery *et al.*, 1997) and Na^+/H^+ exchangers (Baum, 1990, 1992). Similarly, there is a developmental increase in the urinary dilution/concentration capacity of the kidney, with increased activity of the NKCC2 (sodium, potassium, chloride) transporter in the ascending limb of the loop of Henle (Yasui *et al.*, 1996), and also increased number and activity of epithelial sodium channels (ENaCs) in the collecting duct (Huber *et al.*, 1999).

2.3 RENAL MATURATION

Concurrent to, and also continuing beyond the period of nephrogenesis and glomerulogenesis, is the process of renal maturation. Measures of structural maturity in the human fetal and neonatal kidney may be made by assessment of kidney weight and volume. Furthermore, if a known portion of the kidney is available for analysis, full stereological assessment of nephron endowment may be undertaken. It is common following investigations at autopsy, however, that only non-uniform portions of kidney tissue are available for analysis; however, measures of glomerular generation number, nephrogenic zone width, renal corpuscle size, and glomerular maturity may still be made successfully. The final structural maturity of the human kidney (besides ongoing growth by hypertrophy) is not reached until approximately two years of age.

2.3.1 KIDNEY SIZE

An Australian study of fetal growth (in fetuses assessed at autopsy) demonstrated a linear increase in kidney size occurs throughout gestation, with kidney weight increasing from an average of 4.5 g at 20-21 weeks gestation up to 33.3 g at 40-41 weeks (Cussen *et al.*, 1990). The increase in kidney weight remains relative to increases in body weight, with kidney to body weight ratio stable at approximately 0.01 g/g from 20 weeks gestation until term (Mitropoulos *et al.*, 1992).

Unlike *in utero*, renal growth during the early postnatal period is non-linear, with the fastest rate of renal growth occurring during the first few weeks after birth (Zerin and Meyer, 2000). The postnatal increase in kidney length gradually slows until approximately one year of age, when it reaches a stable rate of 2-3 mm/year; after approximately 10 years of age, the rate of renal growth slows further before ultimately ceasing in adulthood (Zerin and Meyer, 2000). In adults, kidneys weigh on average 135 g (McNamara *et al.*, 2008).

2.3.2 NEPHRON ENDOWMENT

Nephron number, the most important measure of renal functional capacity, has been shown through a number of experimental studies to be strongly correlated with renal size

(Gubhaju and Black, 2005; Zohdi *et al.*, 2007; Gubhaju *et al.*, 2009; Sutherland *et al.*, 2009). However, unlike the linear growth of the kidney evident throughout gestation, it has been suggested that the formation of nephrons occurs at varying rates during each stage of development. To date there have been just two studies that have reported changes in fetal nephron endowment throughout gestation (Hinchliffe *et al.*, 1991; Gasser *et al.*, 1993). From the 10^{th} to approximately the 17^{th} week of gestation, Gasser *et al.* (1993) indicated that there is a very gradual increase in the number of nephrons. From approximately the 18^{th} week of gestation onwards, however, nephrogenesis occurs rapidly; this coincides with the period of glomerular arcade formation whereby 60% of nephrons are formed during the third trimester of pregnancy (Hinchliffe *et al.*, 1991). From approximately the 32^{nd} week of gestation, nephrogenesis reaches completion with no new nephrons formed after term birth in the human neonate (Hinchliffe *et al.*, 1991; Gasser *et al.*, 1993).

Recent results from large-scale multi-racial studies have indicated that nephron number in the human kidney can differ 13-fold, ranging from 210,332 to 2,702,079 (Nyengaard and Bendtsen, 1992; Manalich *et al.*, 2000; Hoy *et al.*, 2003; Hughson *et al.*, 2003; Keller *et al.*, 2003; Douglas-Denton *et al.*, 2006; McNamara *et al.*, 2010); however, this is dependent on a large number of both genetic and environmental factors such as birth weight, age, ethnic background, and disease status. Importantly, the full complement of nephrons is achieved prior to term birth; later nephron loss may occur with ageing and the onset of disease.

Another measure of nephron endowment, to be made in circumstances when the full stereological assessment of nephron number is not feasible, is the medullary ray glomerular generation count (Hinchliffe *et al.*, 1992b). This method counts the number of glomeruli (and thus nephrons) visible in a histological section along one side of a medullary ray, from the inner to the outer renal cortex. As with the assessment of nephron endowment, there have been just two previous studies to report changes in the number of glomerular generations with increasing gestational age (Hinchliffe *et al.*, 1992b; dos Santos *et al.*, 2006). There is a significant positive correlation between glomerular generation number and gestational age, whereby the number of generations

increases from about 4 at 24 weeks gestation to approximately 8-10 by term (Osathanondh and Potter, 1963; dos Santos *et al.*, 2006).

2.3.3 NEPHROGENIC ZONE WIDTH

The nephrogenic zone is the area in the outer renal cortex of the kidney which contains developing glomerular structures including comma- and S-shaped bodies. To date, only one research group has published studies regarding the assessment of nephrogenic zone width in the human fetal kidney (dos Santos *et al.*, 2006; Fonseca Ferraz *et al.*, 2008). Results of these studies have shown a significant inverse linear correlation between gestational age and nephrogenic zone width, which decreases from approximately 200 µm at 24 weeks gestation to 100 µm prior to the cessation of nephrogenesis at 32-36 weeks gestation (dos Santos *et al.*, 2006; Fonseca Ferraz *et al.*, 2008). At the completion of nephrogenesis, only mature fully formed glomeruli are present in the superficial cortex, and therefore the measure of nephrogenic zone width is zero.

2.3.4 RENAL CORPUSCLE SIZE

The size of the renal corpuscle in the fetal kidney (cross-sectional surface area, measured by tracing the perimeter of the Bowman's capsule), has been quantified throughout gestation in just two previous large-scale studies (Souster and Emery, 1980; Fonseca Ferraz *et al.*, 2008). Both studies examined differences in renal corpuscle size between inner-, mid- and outer-cortical glomeruli with increasing age. Souster and Emery (1980) determined that there was a decrease in renal corpuscle size between 12 and 20 weeks gestation, after which time the size remained stable until birth. In contrast, however, Fonseca Ferraz *et al*. (2008) demonstrated a significant increase in the size of the mid-cortical glomeruli with increasing gestational age; there was no significant correlation between gestational age and glomerular size evident with the inner- and outer-cortical glomeruli. In general, glomerular size throughout gestation averaged 3,000-5,000 μm^2.

At birth, it was found there was an immediate increase in renal copuscle size to approximately 7,000 μm^2 in the newborn (Souster and Emery, 1980; Moore *et al.*, 1993), and a steady postnatal increase in size has been noted in children examined from birth until 16 years of age where average renal corpuscle size in the oldest subjects measured

approximately 18,000 μm^2 (Moore *et al.*, 1993). These postnatal increases in glomerular size likely relate to the increased functional demand of the kidney as body size increases, and thus glomerular hypertrophy occurs in order to increase glomerular filtration surface area.

Glomeruli in the inner cortex (formed earlier in gestation) are generally larger than those present in the mid- to outer-cortex (Souster and Emery, 1980; Moore *et al.*, 1993). This pattern of glomerular size distribution continues through childhood (Moore *et al.*, 1993); both an increased variation in glomerular volume within the kidney, and an increased mean glomerular volume is strongly associated with a low nephron endowment (Zimanyi *et al.*, 2009; Puelles *et al.*, 2011b).

2.3.5 GLOMERULAR MATURATION

Assessment of renal maturity may also be made histologically via the evaluation of changes in glomerular morphology (Vernier and Birch-Anderson, 1962; Thony *et al.*, 1995; Naruse *et al.*, 2000; Almeida and Mandarim-de-Lacerda, 2002; Lizardo-Daudt *et al.*, 2002; Mishra *et al.*, 2006; Takano *et al.*, 2007). As described previously in Section 2.2.3, individual nephrons develop from a vesicle, through to comma-shaped body followed by an S-shaped body, which are present within the nephrogenic zone of the outer renal cortex. The lower limb of the S-shaped body then differentiates to form the glomerulus, with the most immature form of the renal corpuscle termed as being in the capillary loop stage of development (Naruse *et al.*, 2000). Immunohistochemical analyses of the developing glomeruli, using markers for mesenchymal, endothelial and epithelial cells, have further defined developmental stages according to the location of capillaries, podocytes and mesangium within the glomerular tuft (Naruse *et al.*, 2000; Takano *et al.*, 2007).

Quantification of the changes of glomerular morphology that occur during renal development has previously been reported in just four studies in the human fetus (Vernier and Birch-Anderson, 1962; Almeida and Mandarim-de-Lacerda, 2002; Lizardo-Daudt *et al.*, 2002; Takano *et al.*, 2007), and in two studies in the neonate and infant (Thony *et al.*, 1995; Takano *et al.*, 2007). The majority of these studies have investigated

changes in the V stage (vesicle), C stage (comma-shaped body), S stage (S-shaped body) and M stage (mature/vascularised glomerulus) with increasing gestational age (Almeida and Mandarim-de-Lacerda, 2002; Lizardo-Daudt *et al.*, 2002; Takano *et al.*, 2007). Each report demonstrated a decrease in the proportion of V, C and S stage glomeruli with increasing age; this was concomitant with an increase in the proportion of mature vascularised glomeruli. For example, Takano et al. (2007) showed that the percentage of V stage glomeruli decreased from 60% at 13-19 wks gestation to less than 10% by 35-39 weeks, with a simultaneous increase in mature glomeruli from less than 10% to over 80% by term.

Two studies further differentiated the M stage (mature) glomeruli into subdivisions based on the migration of podocytes within the glomerular tuft (Vernier and Birch-Anderson, 1962; Thony *et al.*, 1995). Throughout gestation, Vernier and Birch-Anderson (1962) demonstrated that the percentage of mature glomeruli increased from 22% at 9 weeks gestation, up to 29% by 19 weeks. Changes in glomerular maturation have not been examined using this criterion during the later stages of gestation. Similarly, however, Thony *et al.* (1995) determined that in the neonatal kidney the percentage of immature glomeruli decreased from birth until approximately 2 years of age, whereby 94-100% of glomeruli were fully matured.

EFFECTS OF PRETERM BIRTH ON RENAL DEVELOPMENT AND FUNCTION

Few studies to date have reported the effects of preterm birth on renal function in the neonatal period, and even fewer have investigated potential underlying structural adaptations in the kidney, such as changes in renal volume and nephron endowment. Given the increasing number of neonates surviving preterm birth, it is important to understand the consequences of preterm birth on the kidney and the implications this may have for long-term renal health.

3.1 RENAL DEVELOPMENT

3.1.1 KIDNEY SIZE

Renal growth, measurable by renal ultrasound or magnetic resonance imaging (MRI), is used by many nephrologists as a predictor of future renal disease (van Venrooij *et al.*, 2010). Importantly, kidney volume has been demonstrated through a number of experimental animal studies to be strongly correlated with nephron number (Zohdi *et al.*, 2007; Gubhaju *et al.*, 2009; Sutherland *et al.*, 2009); others, however, have not found this association (Murawski *et al.*, 2010). Kidney volume is also likely to be highly dependent on factors such as tubular and interstitial mass, renal blood flow, body weight, and glomerular size; therefore, it is often difficult to make predictions of renal functional capacity and nephron endowment based on fundamental structural parameters such as kidney size (Lodrup *et al.*, 2008).

In a recent report by van Venrooij *et al.* (2010), normal postnatal renal growth in preterm neonates (born at less than 31 weeks gestation) was established. Renal length and volume were found to be significantly correlated with both gestational age at birth, and body weight. Huang *et al.* (2007) assessed postnatal renal growth in preterm neonates compared to age-matched control neonates that were assessed soon after birth. This study concluded that the kidney weight to body weight ratio was significantly increased in preterm neonates compared to controls, perhaps reflecting increased renal blood flow or

accelerated postnatal growth following preterm birth. From 31 weeks gestation, however, the renal growth rate was significantly reduced in the preterm neonates compared to the controls. Conversely, Kent *et al.* (2009) found that kidney volume, as measured by MRI, was not different between term-born infants and preterm neonates (born at less than 29 weeks gestation) assessed at term-equivalent age. In the most comprehensive study of renal growth in preterm neonates, involving 466 infants and 1898 ultrasound measurements, Drougia *et al.* (2009) determined that kidney length was significantly decreased in preterm neonates (born 28-34 weeks gestation) compared to those born at term, from three months through to two years of age. Furthermore, in preterm children assessed at term-equivalent age through to 18 months of age, the kidney was of a slimmer shape compared to term-born infants (Schmidt *et al.*, 2005), which potentially reflects decreased radial glomerular generation formation.

3.1.2 NEPHROGENESIS

Human nephrogenesis is normally ongoing until 32-36 weeks gestation *in utero* (Hinchliffe *et al.*, 1991). In preterm infants born before the completion of nephrogenesis, nephron formation may be ongoing following birth in the postnatal environment (Rodriguez *et al.*, 2004; Gubhaju *et al.*, 2009). This is likely a suboptimal environment for renal development to occur (Gubhaju *et al.*, 2011), and may therefore result in impaired renal development and structural alterations in the preterm kidney.

NEPHRON ENDOWMENT

To date there have been only two studies in humans that have assessed glomerular generation number following preterm birth, as a proxy measurement of nephron endowment. In a recent study by Faa *et al.* (2010), which assessed the kidneys of 12 preterm neonates and three term neonates collected at autopsy, glomerular generation number was significantly reduced in the preterm kidneys compared to the controls; however, nephrogenesis had likely not been completed in a number of these neonates. In an earlier autopsy study by Rodriguez *et al.* (2004), preterm infants born at less than 28 weeks gestation were divided into groups according to postnatal age (less than 40 days (short-term survival) or 40 days or more (long-term survival)), and further separated

according to whether renal failure was observed in the neonatal period. The results of this study indicated that glomerular generation number was significantly reduced in all preterm neonates compared to term controls. Glomerular generation number, within the group of infants with a long survival period, was further significantly reduced in the infants with renal failure compared to those with normal renal function. Twenty-five percent of the preterm infants in this study, however, were also IUGR. IUGR is a known cause of impaired renal development (Hinchliffe *et al.*, 1992a; Manalich *et al.*, 2000), and thus likely affected the results of this study.

Glomerular generation number has also been assessed in studies involving a preterm baboon model of preterm birth (Gubhaju *et al.*, 2009; Sutherland *et al.*, 2009); the baboon has been demonstrated to be an ideal model of human renal development (Gubhaju and Black, 2005). The baboon neonates (all born at an appropriate weight for gestational age; AGA) were delivered preterm at a time point equivalent to 27 weeks gestation in humans, and maintained after birth until postnatal day 21. Postnatally, the baboon neonates were cared for in a similar manner to human preterm neonates within a primate neonatal intensive care unit (for a detailed description of the preterm baboon clinical care and treatments, see Thomson *et al.* (2004)). In contrast to the findings of Rodriguez *et al.* (2004), in the preterm baboon kidney there was no difference in glomerular generation number between the preterm neonates and the gestational control and term neonates (Gubhaju *et al.*, 2009). However, it is to be noted that nephrogenesis was still ongoing in the baboon kidney at the time of assessment.

In the preterm baboon model, nephron number was also stereologically assessed using the physical disector/fractionator method (Bertram, 1995). Total nephron number ranged from 193,983 to 334,316 in preterm baboons assessed at postnatal day 21, and 138,078 to 304,186 in term-born baboons; there was no significant difference in nephron endowment between groups (Gubhaju *et al.*, 2009). Importantly, however, nephron density was significantly reduced in the preterm kidney (83,840 nephrons/g) compared to term controls (193,400 nephrons/g) (Gubhaju *et al.*, 2009). As total nephron number was not reduced, and renal corpuscle size was also not affected, this decrease in nephron density in the preterm kidney likely reflects a postnatal increase in tubular and/or interstitial mass.

ABNORMAL GLOMERULAR MORPHOLOGY

Morphologically abnormal glomeruli, exhibiting an enlarged Bowman's space and shrunken glomerular tuft, have been commonly observed in the outer renal cortex of preterm baboon kidneys (Gubhaju *et al.*, 2009; Sutherland *et al.*, 2009). Studies by Gubhaju *et al.* (2009) have reported that the number of abnormal glomeruli varied considerably between individual neonates, ranging from 0.2% to 18% per kidney. Immunohistochemical analyses showed that the affected glomeruli were at an immature stage of development, and that the glomerular tufts were poorly capillarised. An essential growth factor for glomerular capillary development, VEGF, was expressed in the affected glomeruli; however, the level of expression was not quantified (Gubhaju *et al.*, 2009; Sutherland *et al.*, 2009).

Given the immature morphology of the glomeruli, and their localisation to the superficial renal cortex, it is likely that it is newly formed glomeruli that are affected by preterm birth. Furthermore, the difference in the percentage of affected glomeruli between individuals suggests that it may be factors in the postnatal environment (which vary between neonates) that are leading to the glomerular abnormalities rather than preterm birth *per se*. To date, however, the pathogenesis of the glomerular abnormalities is unknown. Given that the grossly abnormal glomeruli are unlikely to be functional, this suggests that there may be a deficit of functional nephrons in the preterm kidney after birth, which in turn may adversely affect the functional capacity of the kidney.

3.2 RENAL FUNCTION

Renal function in the preterm neonate is likely affected by both renal immaturity and also injury during the postnatal period. Despite the range of studies performed to date, standard levels of urinary constituents in the neonate, especially those of the preterm neonate, are not yet clearly defined. It is important for future studies, therefore, to be focused on determining standard values for the assessment of preterm renal function, and also toward the identification of robust biomarkers for the early diagnosis of renal injury in the neonatal period.

3.2.1 SODIUM BALANCE

Studies that have assessed the fractional excretion of sodium during the neonatal period have determined that sodium excretion is significantly higher in preterm neonates compared to term controls (Siegel and Oh, 1976; Aperia *et al.*, 1981a), and it significantly decreases with increasing gestational (Gallini *et al.*, 2000) and postnatal age (Ross *et al.*, 1977; Sulyok *et al.*, 1979; Aperia *et al.*, 1981a; Gallini *et al.*, 2000; Giapros *et al.*, 2007). Interestingly, sodium balance has been shown to be altered very little by nutritional intake, suggesting that a negative sodium balance is unavoidable at birth (Bonsante *et al.*, 2011). With increasing renal maturity, however, a positive sodium balance (low FE_{Na}) is achieved, which is essential for the growth and development of the neonate and the maintenance of fluid homeostasis (Engle, 1986).

3.2.2 GLOMERULAR FILTRATION RATE

During the first week of life following preterm birth GFR is significantly lower in preterm neonates than in term-born controls (Siegel and Oh, 1976; Finney *et al.*, 2000; Schreuder *et al.*, 2009), and is positively correlated with both gestational age at birth, and postnatal age (Clark *et al.*, 1989; Gordjani *et al.*, 1998; Iacobelli *et al.*, 2009). Compared to term neonates, the rate of increase in GFR after birth is slower in neonates born preterm (Bueva and Guignard, 1994; Gordjani *et al.*, 1998). Up until two months of age there are similar findings, with a number of studies observing an increasing GFR concurrent to increasing gestational and postnatal ages (Ross *et al.*, 1977; Fawer *et al.*, 1979; Sulyok *et al.*, 1979; Aperia *et al.*, 1981a; Wilkins, 1992; Bueva and Guignard, 1994; Gallini *et al.*, 2000; Cuzzolin *et al.*, 2006; Thayyil *et al.*, 2008). Although a number of studies have now been performed in this area, there is still a lack of clear definition regarding expected GFR values in the preterm neonate. Recently published standard curves of GFRs in neonates born at 27-31 weeks gestational age, from 7 to 28 days of life, will go some way in aiding in the clinical interpretation of renal function in this particular group (Vieux *et al.*, 2010).

Given that age has been found to be a strong determinant of GFR, the low rate of filtration observed in the preterm neonate after birth is likely primarily resultant from renal immaturity (a low number of functional glomeruli), and is also likely to be

influenced by differences in renal blood flow and vascular resistance. It is essential that GFR is monitored in the postnatal period following preterm birth, as a low GFR may impair renal drug clearance, leading to nephrotoxicity and potentially adverse effects on nephrogenesis (Schreuder *et al.*, 2011).

3.2.3 ACUTE KIDNEY INJURY

The current diagnosis of acute kidney injury (AKI) is based on the RIFLE system, which categorises the stages of increasing AKI severity: Risk, Injury, Failure, Loss and End-stage kidney disease (ESKD) (Bellomo *et al.*, 2004); this system was further modified following recommendations from the Acute Kidney Injury Network (Mehta *et al.*, 2007). The initial clinical indication of AKI risk includes a 50% increase in serum creatinine (or ≥ 0.3 mg/dl within a 48 hour period), and/or a urine output less than 0.5 mg/kg/h for a period of six hours (Bellomo *et al.*, 2004; Mehta *et al.*, 2007). In addition to the changes in urine output and GFR, AKI may also result in proteinuria (Parikh *et al.*, 2010). Importantly, a definition of AKI for use in a neonatal-specific population has not yet been developed.

The causes of AKI in the preterm neonate are primarily pre-renal in origin, arising from conditions which affect renal perfusion such as hypotension, hypoxia and sepsis (Stapleton *et al.*, 1987; Cataldi *et al.*, 2005); these in turn lead to apoptotic, necrotic and inflammatory processes within the kidney (Ueda and Shah, 2000; Bonventre, 2007). AKI is reported to occur in 8% to 24% of preterm neonates admitted to neonatal intensive care units (Stapleton *et al.*, 1987; Hentschel *et al.*, 1996). Importantly, AKI in the preterm neonate may subsequently lead to long-term chronic renal disease (Abitbol *et al.*, 2003).

In a study involving 172 preterm neonates by Cataldi *et al.* (2005), the risk factors for AKI were found to be maternal and neonatal drug administration (NSAIDs and antibiotics, especially ceftazidime), a low Apgar score, and a patent ductus arteriosus. Interestingly, gestational age at birth did not affect risk of AKI; however, the majority of AKI cases (79%) weighed less than 1.5 kg at birth (Cataldi *et al.*, 2005). In a larger study by Cuzzolin *et al.* (2006), involving 281 preterm neonates, a number of risk factors for AKI were also identified. These included maternal NSAID administration, low Apgar score, respiratory distress syndrome, neonatal drug administration (antibiotics and NSAIDs), and clinical

interventions (intubation at birth, catheterization, phototherapy, and mechanical ventilation). The largest study conducted to date was performed by Walker *et al*. (2011); the medical records of 66,526 preterm neonates (born ≤ 30 weeks gestation) from 286 hospitals throughout the USA and Puerto Rico were assessed. In this study, just 4% of neonates had been given the diagnosis of renal dysfunction and/or renal failure. The predominant risk factors for renal dysfunction/failure were low gestational age at birth and low birth weight. Further risk factors included: vasopressor, indomethacin, and antibiotic administration, intraventricular haemorrhage (grade III or IV), a patent ductus arteriosus, necrotising enterocolitis, culture positive sepsis, high frequency ventilation, male gender and non-white race. Importantly, mortality rates were significantly higher in neonates with renal dysfunction and/or renal failure (Walker *et al.*, 2011).

DIAGNOSIS OF ACUTE KIDNEY INJURY

Given the importance of the early diagnosis and treatment of AKI, there has been much recent focus on the discovery of novel urinary biomarkers. In a systematic review of the current literature, Parikh *et al.* (2010) determined that the molecules with the most promise for the diagnosis of established AKI include interleukin-18 (IL-18), kidney injury molecule-1 (KIM-1), N-acetyl-beta-D-glucosaminidase (NAG) and neutrophil gelatinase-associated lipocalin (NGAL); the most encouraging biomarkers for the early diagnosis of AKI also included NGAL, IL-18, fatty acid binding protein (FABP) and cystatin-C.

Urinary NGAL levels have been assessed in preterm neonates with mixed results. Normative values for urinary NGAL in preterm neonates with uncomplicated clinical courses have indicated a greater variation in females than males (Huynh *et al.*, 2009). Studies have also shown that the highest NGAL levels are evident in the neonates that are critically ill, with or without evidence of renal dysfunction (Lavery *et al.*, 2008; Parravicini, 2010). In particular, NGAL has been suggested to be a promising biomarker of late-onset sepsis (Parravicini *et al.*, 2010). Urinary NGAL levels also strongly correlated with gestational and postnatal age (Lavery *et al.*, 2008; Huynh *et al.*, 2009), perhaps reflecting the renal production of NGAL during nephrogenesis (Gwira *et al.*, 2005) which may be still ongoing during the early postnatal period. Further research is required, therefore, in

order to discover a suitable biomarker of AKI for the prediction of renal injury in the preterm neonate.

3.2.4 PROTEINURIA

Proteinuria (the presence of high levels of protein in the urine) may be of glomerular and/or tubular origin. The number of different proteins that have been identified in the human urinary proteome is 1,543 (Adachi *et al.*, 2006); due to the function of the glomerular filtration barrier and tubular reabsorption capabilities, however, proteins are normally only present at very low levels in urine unless renal function is impaired. The presence of proteins with a high molecular weight (HMW) in the urine, such as albumin, is indicative of a disruption in the integrity of the glomerular filtration barrier (Mathieson, 2004). Low molecular weight (LMW) proteins, such as β2-microglobulin, however, pass freely through the glomerulus and undergo reuptake via proximal tubule cells (Tomlinson, 1992; Christensen and Birn, 2001). LMW protein levels in the urine are not routinely measured in the clinical setting, although they may be important markers of tubular cell injury (Rosner, 2009; Parikh *et al.*, 2010).

In the preterm neonate, few studies have been conducted to examine urine protein excretion. In general, the results of these studies have shown a high variability in urine albumin levels between individual neonates (Clark *et al.*, 1989; Fell *et al.*, 1997), with the highest levels exhibited by those with a low gestational age at birth and those that are clinically unstable (Galaske, 1986; Clark *et al.*, 1989; Tsukahara *et al.*, 1994; Fell *et al.*, 1997; Awad *et al.*, 2002). The majority of these studies have only been conducted during the first week of life following preterm birth; however, in a study by Tsukahara *et al.* (1994) urine albumin levels were assessed in preterm and term neonates over the first 28 days of life. Urine albumin levels were found to remain relatively stable postnatally throughout the study period in the preterm neonates, whereas in the term neonate urine albumin was seen to decrease with increasing postnatal age. This result suggests the glomerular filtration barrier following preterm birth is structurally immature, until beyond one month of age. Urinary β2-microglobulin levels have also been shown to be significantly greater in the preterm infant compared to term-born infants throughout the first month of life (Aperia *et al.*, 1981a; Tsukahara *et al.*, 1990; Tsukahara *et al.*, 1994),

and are decreased with increasing gestational and postnatal age (Takieddine *et al.*, 1983). Similarly, levels of α1-microglobulin and retinol binding protein (RBP) are higher in preterm neonates than neonates born at term (Clark *et al.*, 1989; Fell *et al.*, 1997; Awad *et al.*, 2002). To date, however, it remains unclear whether the increased urinary high- and low- molecular weight protein levels reported in preterm babies are associated with renal immaturity and/or renal injury.

3.3 LONG-TERM EFFECTS ON RENAL HEALTH

The majority of studies conducted to date suggest that renal development and function are indeed impaired following preterm birth, which may have significant implications for the long-term renal health of individuals born preterm. In support of this, a number of recent epidemiological studies have strongly linked preterm birth with the development of hypertension, and also renal dysfunction; it is possible that underlying these associations may be a nephron deficit at the beginning of life in individuals born preterm.

3.3.1 CONSEQUENCES OF A REDUCED NEPHRON ENDOWMENT

In recent studies by Rodriguez *et al*. (2004; 2005), it was suggested that preterm birth may lead to impaired nephrogenesis, resulting in a reduced nephron endowment. An adequate number of functional nephrons is deemed to be essential to maintaining optimal renal health throughout life, with a low nephron endowment being strongly linked to both the development of hypertension and renal disease later in life (Hoy *et al.*, 2005; Ingelfinger, 2008).

Nephron number per kidney is highly variable, and can occur in the range of approximately 200,000 to over 2 million in the general human population (Nyengaard and Bendtsen, 1992; Manalich *et al.*, 2000; Hoy *et al.*, 2003; Hughson *et al.*, 2003; Keller *et al.*, 2003; Douglas-Denton *et al.*, 2006; McNamara *et al.*, 2010). The number of nephrons formed is dependent on two main factors: the extent of ureteric bud branching and the ability of the mesenchymal cells to differentiate (Moritz and Cullen-McEwen, 2006). As Shah and colleagues explained (Shah *et al.*, 2004), even a relatively minor decrease in the efficiency of nephrogenesis, through either of these two processes, can lead to a major

deficit in nephron endowment. Severe deficits in nephron number, such as occur in the rare disorder oligomeganephronia, are associated with unavoidable progressive renal failure and subsequent death from uremia at an early age (Suzuki *et al.*, 2005). Although more subtle nephron deficits do not necessarily lead to overt renal dysfunction, there has been considerable evidence put forward to associate a reduced nephron endowment with both an increased susceptibility to renal disease and the development of hypertension (Hoy *et al.*, 2005; Ingelfinger, 2008).

The *Brenner hypothesis* (Brenner and Chertow, 1994) further postulates that a nephron deficit reduces glomerular filtration surface area leading to systemic and glomerular hypertension. In the kidney, remaining nephrons undergo structural and functional adaptations, such as glomerular hypertrophy and permselectivity changes, in order to meet the increasing excretory requirements (Brenner and Mackenzie, 1997). This leads to glomerular hyperfiltration, which renders the glomeruli susceptible to glomerulosclerosis, and in turn a further decrease in filtration surface area occurs; this process thus perpetuates a vicious cycle, eventually resulting in significant nephron loss (Brenner *et al.*, 1988; Brenner and Chertow, 1994). Similarly, a secondary insult to the functional capacity of the kidney, such as occurs with obesity or diabetes, may also overwhelm the compensatory structural and functional changes in the remaining nephrons leading to further nephron loss, a subsequent decline in renal function, and the onset of significant renal disease (Nenov *et al.*, 2000).

HYPERTENSION

There is strong epidemiological evidence to link preterm birth to the development of hypertension in adulthood. In middle-aged adults, there has been found to be a significant inverse correlation between systolic blood pressure and gestational age at birth (Siewert-Delle and Ljungman, 1998; Cooper *et al.*, 2008); a study by Cooper *et al*. (2008) demonstrated that there was a 0.53 mmHg reduction in systolic blood pressure for every one week increase in gestational age at birth. Importantly, the inverse association between blood pressure and gestational age has also been observed in young adults (Kistner *et al.*, 2000; Doyle *et al.*, 2003; Hack *et al.*, 2005; Johansson *et al.*, 2005; Keijzer-

Veen *et al.*, 2005 ; Keijzer-Veen *et al.*, 2007; Lawlor *et al.*, 2007), and even in children that were born preterm (Stevenson *et al.*, 2001; Bonamy *et al.*, 2005; Bonamy *et al.*, 2007).

3.3.2 KIDNEY SIZE, MORPHOLOGY, AND FUNCTION

KIDNEY SIZE

Studies that have assessed kidney size (length and/or volume as determined using ultrasound measurements) in preterm-born children and adults have in general demonstrated a reduction in kidney size compared to individuals that were born at term. Children assessed at primary school age (6-12 years) have in recent studies been shown to exhibit a significant reduction in kidney length and volume (absolute and/or relative to body size) compared to age-matched children that were born at term (Rakow *et al.*, 2008; Zaffanello *et al.*, 2010; Kwinta *et al.*, 2011); however, it is to be noted that there have also been two earlier studies that found no effect of preterm birth on kidney size in children (Vanpee *et al.*, 1992; Rodriguez-Soriano *et al.*, 2005). Interestingly, Zaffanello *et al*. (2010) demonstrated a significant reduction in kidney size in children born at 26-28 weeks gestation, compared to those born at 30-31 weeks gestation; this is the first study to demonstrate that the severity of prematurity has an effect on kidney growth. There has been only one study to date that has examined the effect of preterm birth on kidney size in adulthood. Keijzer-Veen *et al.* (2010) demonstrated that at 20 years of age, following preterm birth at less than 32 weeks gestation, adults had a significantly decreased kidney length and volume (both absolute and relative) compared to individuals born at term; this difference was only statistically significant in females.

RENAL MORPHOLOGY

In a case study of six individuals born preterm (22-30 weeks gestation) and assessed at 15-52 years of age, Hodgin *et al.* (2009) described typical findings of post-adaptive focal segmental glomerulosclerosis (FSGS), including glomerulomegaly, mild effacement of podocyte foot processes, and proteinuria in the absence of nephrotic syndrome. All six patients had no history of other risk factors for FSGS, such as diabetes or long-standing hypertension, which suggests that it may be the preterm birth and/or low birth weight

(possibly leading to impaired renal development) which has predisposed these individuals to renal pathology in adulthood (Hodgin *et al.*, 2009).

RENAL FUNCTION

Low birth weight is strongly linked to the development of renal disease in later life (Hoy *et al.*, 1999; Vikse *et al.*, 2008; White *et al.*, 2009; Benz and Amann, 2010). The effects of preterm birth *per se* (just one of the known causes of low birth weight) on renal function in children and adults has to date, however, only been investigated in a small number of studies with inconclusive results.

In school-aged children, Rakow *et al.* (2008) found no difference in GFR or urinary levels of HMW and LMW proteins in children born at less than 32 weeks gestation, compared to those that were born at term. Similarly, Vanpee *et al.* (1992) determined that there was no difference in renal function in preterm and term-born children at 8 years of age, despite lower GFR and higher urine albumin levels being evident in the preterm group at 9 months of age. Two studies have also been conducted to examine renal function in young adults (20-30 years of age), with both Keijzer-Veen *et al.* (2007) and Kistner *et al.* (2000) finding no effect of preterm birth on GFR or albuminuria.

In contrast, however, a study by Rodriguez-Soriano *et al*. (2005) showed that GFR was significantly reduced in preterm-born children compared to term controls, with impairments in electrolyte excretion also evident. In children examined at 6-8 years of age, Iacobelli et al. (2007) also demonstrated microalbuminuria in 8.3% of the preterm children; this was associated with factors such as neonatal hypotension and increased catch-up growth. Furthermore, in a cohort of 19 year old young adults, those who were born preterm as well as intrauterine growth restricted (IUGR) had a significant reduction in glomerular filtration rates (Keijzer-Veen *et al.*, 2005). Increased risk of renal demise was also evident in individuals born preterm and who were also obese during childhood (Abitbol *et al.*, 2009).

Given these results in preterm-born children and adults, there is some suggestion that preterm birth (particularly in combination with other insults that affect the growth and functional capacity of the kidney) may result in progressive renal failure later in life.

Future research will be required, however, in order to definitively determine the consequences of preterm birth on life-long renal function.

HYPOTHESIS AND AIMS

The focus of this thesis is to examine the effects of preterm birth on renal development and function in the neonatal period, and further to investigate specific factors involved in the postnatal care of the preterm neonate which may affect renal development.

4.1 HYPOTHESIS

As shown in Figure 4.1, it was hypothesised that preterm birth, and related factors involved in the postnatal care of the preterm neonate, would adversely affect renal development and function and lead to renal injury; this thesis is primarily focused on the initial effects of preterm birth on the neonatal kidney. Additionally, it was also hypothesised that preterm birth would ultimately result in a nephron deficit, which in turn would lead to an increased susceptibility to develop renal disease later in life.

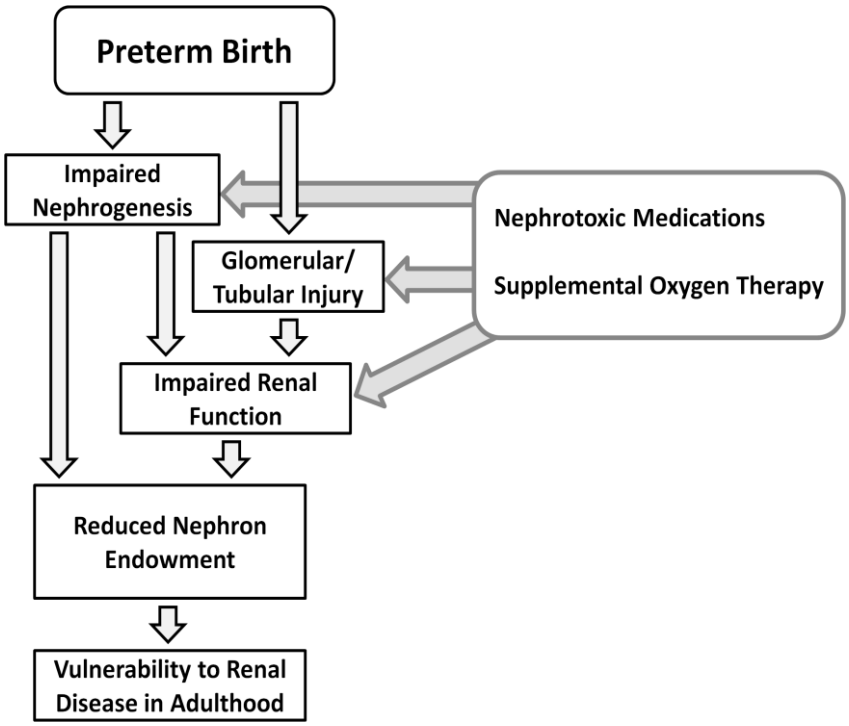

Figure 4.1: Hypothesis flow diagram. It was proposed that preterm birth would impair nephrogenesis and lead to glomerular and/or tubular injury. In turn, these changes would result in impaired renal function in the neonate, and also a reduced nephron endowment. This may be further influenced by exposure to postnatal insults such as supplemental oxygen therapy and treatment with nephrotoxic medications. Ultimately, a nephron deficit would increase the susceptibility of the preterm neonate to developing renal disease later in life.

Specifically, it was hypothesised that preterm birth would impair renal development, leading to a reduced number of glomerular generations compared to aged-matched controls with normal renal development. Furthermore, morphologically abnormal glomeruli, with an enlarged Bowman's space and shrunken glomerular tuft, were likely to be observed in the outer renal cortex of the human preterm kidney. Functionally, it was postulated that following birth preterm neonates would have significantly lower creatinine clearance and higher sodium excretion than neonates born at term. In addition, it was hypothesised that there would be a significantly greater level of urine protein (albumin and β2-microglobulin) and urinary NGAL excretion in preterm neonates compared to term controls. It was also hypothesised that ibuprofen treatment would impair renal development in the early postnatal period. Furthermore, it was postulated that exposure to high oxygen concentrations after birth would adversely affect the development of glomerular capillaries and result a reduction in nephron endowment.

4.2 AIMS

To address these hypotheses, studies in the human preterm neonate (Chapters 2 and 3), as well as in animal models of preterm birth (Chapters 4, 5 and 6) were undertaken. The specific aims of each of these studies are listed below:

Chapter 2: To determine the effects of preterm birth on renal function in the human neonate during the first month of life.

Chapter 3: To determined the effects of preterm birth on postnatal renal development and renal morphology in the human neonatal kidney.

Chapter 4: To determine the effects of preterm birth on glomerular capillary length and surface area in the preterm lamb kidney.

Chapter 5: To determine the effects of neonatal hyperoxia exposure on renal development in a mouse model.

Chapter 6: To determine the effects of neonatal ibuprofen treatment on renal development in a preterm baboon model.

4.3 STUDY DESIGN

The first two experimental chapters (Chapters 2 and 3) are focused on describing the effects of preterm birth on renal development and function in human neonates during the early neonatal period. These studies were initially commenced by another Ph.D student in our laboratory, Dr. Lina Gubhaju, with the preliminary findings of the research presented in her Monash University Ph.D thesis entitled *The Effect of Preterm Birth on the Kidney* (Gubhaju, 2009); in the current thesis, these studies have been completed and the final manuscripts have been generated (Chapters 2 and 3).

In the first experimental chapter (Chapter 2), preterm neonates were prospectively recruited for the assessment of renal function (creatinine clearance, the fractional excretion of sodium, and urinary albumin, β2-microglobulin, and NGAL excretion) from postnatal day 3 to day 28. Indices of renal function in these neonates were compared to normal renal function in term-born neonates during the first month of life. Chapter 3 describes an assessment of renal structural development in the human neonate following preterm birth; kidneys collected at autopsy in preterm neonates (24-35 weeks gestational age, 2-42 days postnatal survival) were compared to those of postconceptional age-matched controls to determine whether preterm birth affects the normal growth trajectory of the kidney. Furthermore, whether morphologically abnormal glomeruli were observed in the human preterm kidney (as has been commonly seen in the baboon kidney following preterm birth) was also determined. Similarly, Chapter 4 also focuses on the effects of preterm birth on the structural development of the kidney. Using a preterm lamb model, in which the lambs were delivered moderately preterm and ventilated for three days after birth, the length and surface area of the glomerular capillaries was assessed.

Two factors involved in the postnatal care of the preterm neonate that may potentially influence renal development were assessed in Chapters 5 and 6; by using carefully controlled animal studies, many of the confounding factors associated with studies in human infants can be eliminated. In Chapter 5, the effects of neonatal hyperoxia exposure on the developing kidney were investigated in a neonatal mouse model (where nephrogenesis is ongoing postnatally for approximately 7 days following birth).

Supplemental oxygen therapy is a common treatment for the preterm neonate in order to raise blood oxygen levels in underdeveloped lungs; to determine whether hyperoxia exposure would affect renal development, glomerular maturity, nephron endowment and glomerular size were assessed. In Chapter 6, a clinically relevant preterm baboon model was used to determine the effects of early postnatal ibuprofen administration on renal development following preterm birth; ibuprofen (a NSAID) is commonly administered to preterm neonates in order to treat a patent ductus arteriosus, and is known to have adverse effects on renal function.

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REVIEW 1:

PRETERM BIRTH AND THE KIDNEY: IMPLICATIONS FOR LONG-TERM RENAL HEALTH

CHAPTER ONE (REVIEW 1) DECLARATION

In Review 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Wrote some sections	20%

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution (%) student co-authors only
Lina Gubhaju	Wrote majority of sections	70%
M. Jane Black	Assisted in writing some sections	10%

Candidate's Signature [redacted] **Date** 30/04/12

Declaration by co-authors
The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) **Department of Anatomy & Developmental Biology, Monash University**

	Signature	Date
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Preterm Birth and the Kidney: Implications for Long-Term Renal Health

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Abstract

Although the majority of preterm neonates now survive infancy, there is emerging epidemiological evidence to demonstrate that individuals born preterm exhibit an elevated risk for the development of hypertension and renal impairment later in life, thus supporting the developmental origins of health and disease hypothesis. The increased risk may potentially be attributed to a negative impact of preterm birth on nephron endowment. Indeed, at the time when most preterm neonates are delivered, nephrogenesis in the kidney is still ongoing with the majority of nephrons normally formed during the third trimester of pregnancy. A number of clinical studies have provided evidence of altered renal function during the neonatal period, but to date there have been limited studies describing the consequences of preterm birth on kidney structure. Importantly, studies in the preterm baboon have shown that nephrogenesis is clearly ongoing following preterm birth; however, the presence of abnormal glomeruli (up to 18% in some cases) is of concern. Similar glomerular abnormalities have been described in autopsied preterm infants. Prenatal and postnatal factors such as exposure to certain medications, hyperoxia and intrauterine and/or extrauterine growth restriction are likely to have a significant influence on nephrogenesis and final nephron endowment. Further studies are required to determine the factors contributing to renal maldevelopment and to identify potential interventional strategies to maximize nephron endowment at the start of life, thereby optimizing long-term renal health for preterm individuals.

Keywords

preterm birth, kidney, nephrogenesis

Introduction

Over the past 2 decades, both the incidence of preterm birth and survival following preterm birth have substantially increased in developed countries.[1,2] Preterm birth is associated with an increased risk of morbidity and mortality in the neonatal period and this is largely attributed to the underdevelopment of vital organs, in particular those organs that undergo marked growth and maturation during late gestation. Although there has been considerable research into the effects of preterm birth on development of the lungs and strategies implemented, considerably less is known of the effects of preterm birth on the kidneys. This is important because nephrogenesis (the formation of nephrons) predominantly occurs in late gestation at a time when most preterm infants are likely to be delivered. Given that a reduced nephron endowment is linked to the etiology of hypertension and susceptibility to renal disease,[3] it is imperative to gain an understanding of how preterm birth affects renal structural and functional development. Indeed, there is now convincing epidemiological evidence to demonstrate that the antecedents of adult disease can often originate in early life,[4-6] and in support of the developmental origins of health and disease hypothesis there is substantial recent epidemiological evidence linking preterm birth with an increase in blood pressure in later life[7-15]; this may be a consequence of a reduced nephron endowment in individuals born preterm.

This review provides an overview of the current knowledge relating to the effects of preterm birth on renal development and function in the neonate and the implications for long-term renal health. Key directions for future research are also identified.

Preterm Birth

Preterm birth (birth prior to 37 complete weeks of gestation) can be subclassified depending upon severity: very preterm (<32 weeks gestation) and extremely preterm (<28 weeks gestation).[16,17] Survival of preterm neonates, particularly those

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born extremely preterm, has also improved substantially through the 1990s such that neonates born at 25 weeks of gestation now have a chance of survival as high as 79%.[18] At the time when most preterm neonates are delivered, many of the vital organs are still undergoing rapid development and maturation. In particular, nephrogenesis in the human kidney is not complete until 34 to 36 weeks of gestation.[19] Furthermore, renal insufficiency is commonly observed in preterm neonates, with the incidence of renal failure in infants admitted to neonatal intensive care ranging from 8% to 24%.[20-23]

Kidney Development

Development of the permanent kidney, the metanephros, begins at approximately day 30 of gestation with the outgrowth of the ureteric bud from the Wolffian duct.[24] Subsequent events include invasion of the mass of metanephric mesenchyme by the ureteric bud, followed by reciprocal inductive interactions that leads to the dichotomous branching of the ureteric bud and the formation of nephrons at the ureteric bud tips.[25] Importantly, the majority of nephrons are formed from week 20 until 34 to 36 weeks of gestation.[19,26] For a comprehensive review of mammalian kidney development, refer to Moritz et al.[25,27]

Coinciding with the formation of the first nephrons, fetal urine production begins at approximately 9 to 10 weeks of gestation and this forms a significant component of the amniotic fluid volume.[28-31] In utero, renal vascular resistance is high and both renal blood flow and glomerular filtration rate (GFR) are low.[29,31] Tubular reabsorption begins by week 12 to 14[31]; in general, the fetal renal tubules excrete large amounts of sodium.[28] At birth, there is an increase in cardiac output, renal blood flow, and a decrease in vascular resistance, thereby increasing GFR.[32-34] In term newborns, GFR has been shown to double during the first 2 weeks of life.[35,36]

The Effect of Preterm Birth on Nephrogenesis

Given that nephrogenesis is still ongoing prior to ~34 weeks of gestation, it is likely that there will be alterations in the structural development of the kidney in neonates born at less than 34 weeks of gestation. These potential structural changes have to date been studied by examining kidney size.[37-40] Further morphometric analyses have also been undertaken to determine whether there is a reduction in nephron endowment following preterm birth.[41-43]

Kidney Size

An advantage of using ultrasound and MRI to measure kidney volume and length in human participants is that it can be done *in vivo*. For example, Huang et al[37] examined kidney volumes of 56 preterm neonates born prior to 34 weeks of gestation at 14 to 96 days after birth. The study found that absolute kidney volumes were significantly larger in preterm neonates with a postconceptional age (defined as the sum of gestational age and postnatal age in weeks) equivalent to less than 31 weeks of gestation (a time-point when nephrogenesis would most likely still be ongoing) compared to controls (comprised of neonates with a gestational age at birth ranging from 28 weeks to 40 weeks) that were examined within 48 hours after birth. This increase in absolute kidney size may be due to the greater functional demand on the neonatal kidney leading to glomerular hypertrophy and hyperfiltration following preterm birth. Conversely, neonates with a postconceptional age equivalent to 31 weeks of gestation or greater (a time-point when nephrogenesis would have ceased or is close to ceasing) had significantly smaller absolute kidney volumes compared to controls. In contrast, however, Kent et al[39] recently reported no differences in absolute kidney volume or kidney volume relative to body weight in preterm neonates (born at 25-28 weeks gestation) compared to term-born controls. MRI measurements were undertaken once the neonate reached term-corrected age (age equivalent to 37-40 weeks of gestation) in the preterm neonates and within the first 4 weeks of life in the term newborns. In an older cohort of infants, Schmidt et al[40] found that relative kidney volume was significantly smaller in preterm infants (born at less than 37 weeks gestation) at 3 months of term-corrected age compared to 3-month-old term infants. The differences observed between studies are most likely attributed to the varying postnatal ages at the time of assessment.

To date, a study by Keijzer-Veen et al[44] is the only study that has examined renal size in preterm individuals in adulthood. In that study, 20-year-old adult preterm individuals (born at less than 32 weeks of gestation) had significantly smaller absolute and relative left kidney lengths and volumes compared to 20-year-old term controls; the difference was only significant in females.[38]

To date, there have been very few studies that have examined the effect of preterm birth on the morphometric structure of the kidney. In this regard, it is important to gain an understanding of whether preterm birth adversely affects nephron number, as this has important implications for long-term renal health.

Glomerular Generation Number

The medullary ray glomerular generation counting method[43,45-47] is a useful index of renal maturity and nephron endowment that can be utilized in the analysis of nonuniform portions of the kidney which are collected at autopsy. To date, there have been only 2 published human studies (apart from case studies) investigating the effect of preterm birth on postnatal nephrogenesis. In a study by Rodriguez et al,[43] the number of glomerular generations was compared between term and preterm infants[44]; compared to term-born infants, the number of glomerular generations in the kidney was significantly reduced in all of the preterm groups. It is important to note when interpreting the data from this study, however, that many of the infants in the preterm groups were also intrauterine growth-restricted (IUGR). Therefore, it is difficult to ascertain whether the reduction in glomerular generations observed in

the preterm infants is due to IUGR or preterm birth *per se*. A recent study by Faa et al[41] also found marked interindividual variability in the number of glomerular generations in kidneys from autopsied preterm neonates. It was not described in this study whether any of the preterm neonates were also growth-restricted.

Nephron Number Estimation in a Nonhuman Primate Model of Preterm Birth

Due to the complexity of the postnatal care of the preterm human infant and the confounding factors associated with human autopsy studies, it is important to undertake controlled animal studies in an appropriate model to examine the effects of preterm birth on nephrogenesis. We have previously shown that the baboon is an ideal model to study human nephrogenesis since it very closely matches that of the human, with nephrogenesis ceasing prior to term by 175 days gestation (term = 185 d gestation).[48] Appropriate for gestational age (AGA) baboons were delivered preterm (125 days of gestation). This time-point is equivalent to approximately 27 weeks gestation in humans and considered to be extremely premature.[42,49] Following birth, the baboon neonates were ventilated in the neonatal intensive care unit for a maximum period of 21 days.

Similar to findings in preterm neonates,[37] kidney weight and volume relative to body weight were significantly higher in the preterm baboon neonates compared to gestational age-matched controls, suggesting a greater functional demand on the neonatal kidney following the transition from the intrauterine to the extrauterine environment as well as the increased demand of postnatal growth. Nephrogenesis was found to continue in the extrauterine environment following preterm birth, with structural evidence of ongoing nephrogenesis (ureteric bud, metanephric mesenchyme, Comma- and S-shaped bodies in the outer renal cortex) accompanied by a significant increase in the number of glomerular generations and nephron number by postnatal day 21.[42] Total nephron number ranged from 193 983 to 334 316 in baboons born at term and 138 078 to 304 186 in preterm baboons at postnatal day 21. There was a significant decrease in glomerular density (glomeruli/gram) in the preterm kidneys compared to the gestational controls suggestive of altered renal growth and potentially an increase in tubular mass. Nephron number was estimated using unbiased stereology, the gold standard method for the determination of nephron number.[50] However, the small sample size and wide variation in the nephron number results due to biological variability[51] must be noted when interpreting the nonhuman primate studies.

Preterm Birth Increases the Risk of Abnormal Glomerular Development

Alarmingly, abnormal glomeruli with a dilated Bowman's space and shrunken glomerular tuft (Figure 1) were often present in the superficial renal cortex of kidneys from the preterm baboons (ranging from 0.2% to 18% per kidney).[42,52]

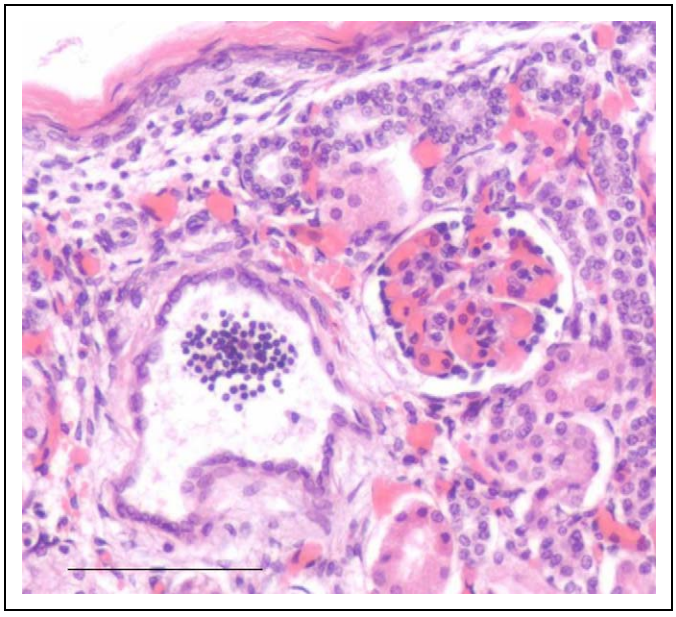

Figure 1. Representative photomicrograph of a preterm human kidney, exhibiting an abnormal glomerulus with an enlarged Bowman's space and shrunken glomerular tuft. These abnormal glomeruli were only found in the outer renal cortex of preterm human kidney suggesting that they were formed in the extrauterine environment. Scale bar = 100 μm.

Importantly, similar abnormal cystic glomeruli have been observed in the outer cortex of autopsied preterm kidneys.[53] The localization of these abnormal glomeruli to the outer cortex strongly suggests that the abnormalities are present only in glomeruli that were newly formed following preterm delivery; therefore, glomeruli formed in the extrauterine environment appear to be "at risk." The variability in this proportion of abnormal glomeruli in preterm kidneys may be attributed to differences in the postnatal care of the preterm neonate. However, to date, no clear link between postnatal management and the number of abnormal glomeruli has been determined.[52]

Immunohistochemical analyses indicated that the tuft of the most grossly abnormal glomeruli was composed primarily of podocytes, suggestive of a relatively immature stage of development.[42,52] Furthermore, abnormal glomeruli were poorly vascularized; we postulate that these poorly vascularized glomeruli would never have become functional. Given that in some kidneys up to 18% of glomeruli were abnormal, this subsequent reduction in the endowment of functional nephrons will likely have adverse consequences on renal function in the preterm neonate.

Renal Function in Preterm Neonates

In the literature, studies of renal function in preterm neonates have varied in the postnatal time-points examined, ranging from the first few days following birth,[54-58] several weeks following birth,[35,36,59] to several months following birth.[60-62] Studies conducted in clinically stable preterm neonates demonstrate that plasma creatinine significantly correlates with

gestational age such that it is highest in the most preterm neonates and decreases gradually with increasing postnatal age.[36,55,56] Similarly, studies have also shown that creatinine clearance (CrCl; an estimate of GFR) increases with increasing gestational age at birth and increasing postnatal age.[36,61] The fractional excretion of sodium, a measure of tubular function, is inversely related to gestational age at birth and decreases with postnatal age.[36,61]

Acute renal failure, defined as a sustained extreme decline in creatinine clearance ranges in incidence from 8% to 24% in preterm neonates.[63] Causes of renal failure include renal hypoperfusion, asphyxia, respiratory distress syndrome, and maternal and neonatal exposure to nephrotoxic medications such as antibiotics and indomethacin (a nonsteroidal anti-inflammatory drug [NSAID]).[23] This is particularly relevant to the extremely preterm infants who encounter greater morbidity and are thus administered a number of potentially nephrotoxic drugs which may lead to renal failure.[23,64,65]

Novel Methods to Examine Renal Function in Preterm Neonates are Required

It is important to note that creatinine levels (used to estimate GFR in preterm neonates) are strongly influenced by maternal creatinine levels in the first few days after birth and they are also highly dependent on muscle mass. Furthermore, creatinine clearance requires a timed urine collection and corresponding serum measurement, which is often difficult to obtain in the clinical setting. Serum cystatin C has also been suggested to be a useful and accurate measure of GFR in preterm neonates since it is filtered completely by the glomerulus and is not affected by muscle mass.[66-68] However, obtaining serum samples from preterm neonates, particularly the extremely preterm neonates, is often difficult. In terms of tubular function, estimation of the fractional excretion of sodium is influenced by factors such as sodium intake and sodium supplementation, which often vary between neonates. Therefore, other clinical measures of glomerular and tubular function are necessary for an accurate and efficient evaluation of renal function in preterm neonates.

Measurement of high-molecular weight proteins (albumin) and low-molecular-weight proteins (β2-microglobulin, α1-microglobulin, and retinol-binding protein) in urine are useful assessments of glomerular[69-71] and tubular[72] function, respectively; however, to date there has been limited studies undertaken in preterm neonates. In utero, albumin, β2-microglobulin, and α1-microglobulin content in amniotic fluid have been shown to decrease significantly with increasing renal maturation.[73,74] Due to renal immaturity during the neonatal period, it has been shown that neonatal urinary protein content is also elevated compared to adults.[75,76]

Based on current findings, urinary albumin levels appear to remain elevated in preterm neonates compared to term neonates, with levels increasing with decreasing gestational age and also in the setting of neonatal illnesses.[56,77-80] For example, a study by Tsukahara et al[77] found that urinary albumin levels remained high throughout the first month of life in moderately preterm neonates, whereas in term newborns, urinary albumin levels gradually decreased during the first month of life. Urinary albumin levels also appear to be higher with decreasing gestational age at birth.[79] For example, Fell et al[79] reported that urinary albumin levels were significantly elevated on the first day of life in neonates born extremely preterm (24 – 28 wk gestation) compared to term and preterm infants delivered after 29 weeks of gestation.

Urinary levels of low-molecular-weight proteins are also significantly elevated in preterm neonates compared to full-term neonates.[35,56,75,77,78,81] For instance, Tsukahara et al[77] found that after birth, β2-microglobulin levels reached a peak on day 7 of life and gradually decreased; however, levels remained significantly elevated throughout the first month of life in preterm neonates compared to term neonates. To date, it remains unclear whether the increased urinary high- and low-molecular weight protein levels reported in preterm neonates relates to renal immaturity and/or injury.

Urinary NGAL: An Emerging Biomarker of Acute Renal Injury in Preterm Neonates

Neutrophil gelatinase-associated lipocalin (NGAL) has also been previously used as an early indicator of intrinsic renal injury following ischemia and nephrotoxic exposure in animals[82] and following cardiopulmonary bypass and renal transplantation in adults and children.[83] Recently, there have been 3 studies that have examined urinary NGAL as a measure of renal function in preterm neonates. Refer to Parravicini[84] for a comprehensive review of the clinical utility of urinary NGAL in the neonatal setting. To date, levels of urinary NGAL have been found to correlate with gestational age and birth weight in preterm neonates, with those in the lower birth weight categories demonstrating greater variability.[85] Furthermore, reference values of urinary NGAL in clinically stable preterm neonates without risk factors for renal impairment have been established.[83,86]

Future research must be directed toward utilizing urinary NGAL in the clinical setting to identify and/or predict renal injury in preterm neonates. Studies thus far suggest that there is wide variability amongst preterm neonates in both the levels of urinary protein and urinary NGAL; it is therefore important to determine the potential prenatal and/or postnatal factors contributing to this variability.

Factors That May Influence the Development and Function of the Preterm Kidney

There are a number of prenatal and postnatal factors associated with preterm birth that are likely to have an adverse effect on the developing kidney and which may account for the variability in glomerular abnormalities observed in the preterm kidney. These include intrauterine and extrauterine growth restriction, exposure to antenatal medications/glucocorticoids, postnatal exposure to a hyperoxic environment, and the administration

of nephrotoxic medications. The consequences of some of these prenatal and postnatal factors have been well described in the literature, whereas others require further research.

Intrauterine and Extrauterine Growth Restriction

A significant proportion of preterm infants are IUGR, with poor growth of the IUGR fetus linked to the induction of preterm delivery.[87] It is conceivable that the IUGR preterm neonate will be more vulnerable to renal insults since it has been well established that IUGR leads to a low nephron endowment[88-90] and renal dysfunction.[91] A study by Drougia et al[92] suggested that kidney length (corrected for body weight and surface area) remains smaller for up to the second year of life in preterm neonates that were also small-for-gestational age (SGA) compared to those that were an appropriate size for gestational age (AGA). In contrast, Keijzer-Veen et al[38] found no differences in absolute or relative renal size between AGA and SGA preterm adults. However, in another study, Keijzer-Veen et al[93] reported lower GFR and higher urinary albumin excretion in adults born extremely preterm who were also SGA, compared to those born preterm and AGA. These studies suggest that renal size may not always be reflective of renal function.

In general, preterm infants do not achieve the normal rate of growth *ex utero* as that *in utero*, therefore resulting in extrauterine growth restriction (EUGR).[94,95] Extrauterine growth restriction in premature neonates (defined as growth below the 10th percentile of intrauterine growth expectation[95]) is also likely to have significant implications for ongoing nephrogenesis and consequently on renal function in adult life. In this regard, Bacchetta et al[96] demonstrated lower GFRs (albeit in the normal range) in very preterm (<30 weeks gestation) children examined at 7 years of age that were either IUGR or EUGR, compared to children with appropriate prenatal and postnatal growth. This is the only study to date that has demonstrated the importance of adequate postnatal growth of the preterm neonate in determining later renal functional capacity. Future research in this area is essential in order to ascertain ways in which to achieve optimal renal development in the NICU setting. Potentially, renal size can be measured postnatally in living preterm neonates to compare renal growth in neonates that have grown appropriately to those that have been growth-restricted *ex utero*. Furthermore, longitudinal studies examining both renal function and size in EUGR preterm individuals in adulthood will be necessary in order to determine whether these observations persist into adulthood.

Antenatal Glucocorticoids

Improved survival of preterm neonates can be largely attributed to the use of antenatal glucocorticoids.[97] Studies have shown that antenatal glucocorticoids increase renal functional maturation by increasing mean arterial pressure, renal blood flow, and GFR,[30,34,98-102] suggesting that nephrogenesis may also be affected. Rodent and ovine studies, however, have demonstrated adverse effects of glucocorticoid exposure during early to mid pregnancy, resulting in fetal growth restriction[103] and reductions in nephron number;[104-106] earlier exposures near the commencement of nephrogenesis had the most adverse effects.[105,107-109] In this regard, Ortiz et al[105] found that maternal administration of 2 doses of dexamethasone on either embryonic days 15 and 16 or on days 17 and 18 of gestation in the rat leads to a 20% and 30% reduction, respectively, in nephron number in the offspring. Additionally, a recent study by Zhang et al[104] in the ovine model demonstrated a reduction in nephron number in response to 2 doses of antenatal steroids administered at 80 and 81 days of gestation (0.55 of gestation). Interestingly, in a nonhuman primate model of preterm birth, exposure to antenatal glucocorticoids (6 mg betamethasone 48 and 24 hours prior to preterm birth) during late gestation (0.67 of gestation) led to renal hypertrophy and an increase in the number of developed glomeruli in the kidney compared to preterm neonates that were not exposed to antenatal glucocorticoids.[42] The increase in the number of developed glomeruli is most likely suggestive of accelerated maturation of developing glomeruli, which is in accordance with previous studies demonstrating that glucocorticoids induce organ maturation.[110] The differences in findings between studies may be indicative of differential effects of antenatal glucocorticoids according to the timing of exposure and/or the dose of steroids administered. It must be noted that the dose of antenatal glucocorticoids administered to the pregnant baboons ($\sim$0.4 mg/kg), although physiologically relevant to the baboon,[111,112] is higher than the dose used in rodent (0.2 mg/kg)[105] and sheep studies (0.17 mg/kg)[104,106] and also the currently recommended dose given to pregnant women at risk of preterm delivery ($\sim$0.2 mg/kg for a 60 kg woman).[113] As discussed earlier, it is also important to note the small sample size in the nonhuman primate studies (4 kidneys per group); in future studies, it is important to repeat these experiments in a larger sample size in order to confirm the findings.

Neonatal Medications

Renal function in the preterm neonate can be further hindered by the administration of potentially nephrotoxic medications during the neonatal period at a time when nephrogenesis is still ongoing. Cataldi et al[65] undertook a case control study which demonstrated an increased prevalence of acute renal failure in those neonates who received more drugs in the first few days of life (antibiotics, NSAIDs, and diuretics) as well as those mothers that received more drugs (antibiotics and NSAIDs) during pregnancy.

Aminoglycoside Antibiotics. Preterm infants are often exposed to antibiotics *in utero* and/or immediately after birth, which could adversely affect nephron formation. Aminoglycosides (the most commonly administered antibiotic) readily cross the placenta and, importantly, their administration to guinea pig and rat dams has been shown to lead to oligonephronia in the offspring.[114] Importantly, it has been shown that when high levels of gentamicin accumulate in the kidney, particularly

in the proximal tubules, this can lead to tubular cell necrosis.[115,116] Furthermore, in experimental metanephric organ culture studies, incubation of the developing kidney with gentamicin has been shown to lead to decreased branching morphogenesis of the ureteric tree and thus reduced nephron formation.[117,118] Clinical studies have also reported that postnatal gentamicin exposure leads to a marked increase in the fractional excretion of sodium[119,120] and a decrease in GFR[121-127] as well as an increase in urinary protein excretion in preterm neonates.[122,128,129] Therefore, it is essential to determine whether postnatal administration of nephrotoxic antibiotics to the preterm neonate is contributing to a reduced nephron endowment and consequently reduced renal function later in life.

Nonsteroidal Anti-Inflammatory Drugs. Other commonly prescribed therapeutic medications in preterm neonates, particularly those born extremely preterm, include the NSAIDs, which are prostaglandin inhibitors, administered in order to close a patent ductus arteriosus.[130,131] Nonsteroidal anti-inflammatory drugs are also used as tocolytic agents, which are administered to pregnant women in order to delay preterm labour[132] or to treat polyhydramnios.[133] Nonsteroidal anti-inflammatory drugs, such as indomethacin and ibuprofen, are known to be detrimental to the kidney, particularly the developing kidney.[134] In the preterm neonate, administration of NSAIDs has been shown to delay the normal increase in GFR that occurs after birth and lead to a decrease in urine output.[64,125,135,136] Importantly, in some cases, prolonged exposure to antenatal NSAIDs has been linked with oliguria, anuria, and/or renal failure in the neonate; these functional changes were accompanied by the presence of cystic lesions in the kidney.[137-139] It is important to note, however, that not all cases of antenatal NSAIDs exposure have led to adverse effects on the neonatal kidney, suggesting that there were underlying factors that rendered some neonates more vulnerable.

An experimental study by Kent and colleagues[140] demonstrated podocyte foot process effacement in rat offspring exposed to maternal indomethacin or ibuprofen from embryonic day 16 to 20, with higher doses resulting in more severe pathology.[140] Postnatal administration of NSAIDs (during the period of active nephrogenesis in the rat) also led to podocyte foot process effacement and renal interstitial edema. Gentamicin administration in combination with indomethacin resulted in the most severe pathology; however, there was no effect on absolute glomerular number measured at day 14 of life. Since the severe glomerular damage was observed, however, these results do not exclude the possibility of future glomerular loss.[141]

Hyperoxia. Postnatal exposure to hyperoxia in the preterm neonate is linked to the development of both bronchopulmonary dysplasia and retinopathy of prematurity; underlying each of these conditions is impaired angiogenesis.[142,143] Results of a recent study suggest, however, that microvascular rarefaction may not only be confined to the retina and lung, with children born very preterm ($\leq$30 weeks gestation) demonstrating significantly reduced functional skin capillary density at 7 to 12 years of age.[144] Whether glomerular vascularization following preterm birth is also adversely affected by hyperoxia in the extrauterine environment has not been examined.

The kidney is a highly vascular organ, with ongoing vascularization of the glomeruli occurring following preterm birth. During nephrogenesis *in utero*, the mammalian fetal environment is relatively hypoxic;[145-147] rat metanephric organ culture studies have shown low (1%-3%) oxygen concentrations to be optimal for both vasculogenesis and tubulogenesis.[148] At birth, preterm infants are exposed to atmospheric (21%) oxygen levels, and this physiologic hyperoxia may be further augmented by the use of supplemental oxygen during the neonatal period. Therefore, it is conceivable that development of the glomerular capillaries will be adversely affected by high oxygen concentrations following preterm delivery.

To date, there has been just one experimental study reporting the effects of neonatal hyperoxia on the adult kidney. Interestingly, Yzydorczyk et al[149] demonstrated that nephron endowment was reduced by 25% in adult rats (25-35 weeks of age) exposed to hyperoxia during the period of postnatal nephrogenesis. However, since the rats were also found to have increased blood pressure by 9 weeks of age, it cannot be determined from this study whether the nephron deficit was the result of the exposure to hyperoxia or induced by the prolonged hypertension.

Long-Term Consequences of Preterm Birth on the Kidney

There is strong epidemiological data linking low birth weight with an increased risk of adult-onset diseases including renal disease.[7-15] To date, this association has been most evident in individuals born of low birth weight as a result of IUGR rather than preterm birth. However, there are recently emerging epidemiological data linking gestational age at birth and blood pressure in young adult men and women. For example, Cooper et al[8] found a 0.53 mm Hg reduction in systolic blood pressure, in middle-aged individuals, for every 1 week increase in gestational age at birth. This is important, since it has been shown in population-based studies that a 2 mm Hg reduction in diastolic blood pressure in adult life results in a 6% reduction in the risk of coronary artery disease and a 15% reduction in the risk of stroke or transient ischemic attack.[150]

In some studies, renal function in school-aged preterm children, and in 20-year-olds has been shown to be similar to renal function of participants born at term.[13,151-153] To the contrary, Rodriguez-Soriano et al,[154] however, found that GFR was significantly lower in 8–year-old children that were born preterm. Therefore, further studies are required to make definitive conclusions on the effects of preterm birth on renal function later on in life, and the factors that may be associated with abnormal renal function observed in some studies. In this regard, there is emerging evidence that obesity increases the risk of kidney dysfunction in preterm children. Levels of proteinuria and albuminuria were shown to be significantly

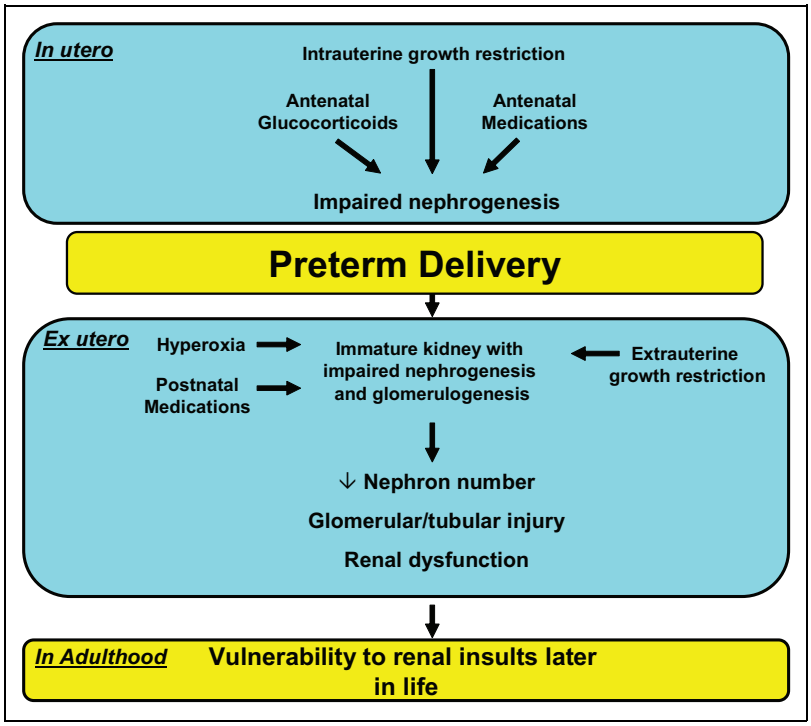

Figure 2. Depiction of the prenatal and postnatal factors that may contribute to impaired nephrogenesis and glomerulogenesis in the preterm kidney. These factors individually, or in combination, are likely to have an adverse impact on nephrogenesis, lead to a nephron deficit, and therefore render the kidney vulnerable to injury later in life.

greater in an obese preterm cohort than in a nonobese preterm cohort.[155]

The only published study describing renal pathology in preterm individuals is a case-study of 6 individuals (age 15-52 years) born at 22 to 30 weeks gestation with extremely low birth weight.[156] Secondary focal segmental glomerulosclerosis in 8.8% of glomeruli (range 5%-16%) was reported in 4 of 5 patients showing a predominance of globally sclerotic glomeruli. Other pathological features included glomerulomegaly and mild foot process effacement. Importantly, all patients had normal renal function and did not have a history of diabetes or long-standing hypertension, suggesting that preterm birth and/or low birth weight may be a predisposing factor in the development of renal pathology.

Can Nephrogenesis be Stimulated in the Preterm Neonate?

Given the long-term adverse consequences of a low nephron endowment, it is important to determine if nephrogenesis can be stimulated in the preterm neonate to potentially prevent the development of disease later in life. As depicted in Figure 2, prenatal and/or postnatal factors associated with preterm birth, such as IUGR and EUGR, exposure to maternal glucocorticoids *in utero*, and exposure to antibiotics and other medications either *in utero* or postnatally, are likely to have an adverse impact on nephrogenesis, thus leading to a nephron deficit and subsequently rendering the kidney vulnerable to secondary insults. The question thus arises: are there any interventional strategies that could be utilized to stimulate nephrogenesis in the immediate postnatal period that would enhance nephron endowment in the preterm infant and ultimately lead to improved renal function later in life?

There is a large body of experimental studies, both *in vivo* and *in vitro*, linking the administration of retinoic acid (the active metabolite of vitamin A) with stimulation of nephrogenesis in the fetal kidney.[157,158] However, this enhancement of nephrogenesis did not occur when retinoic acid was administered postnatally to prematurely delivered baboons[52]; this probably relates to the timing of the retinoic acid administration (after the period of branching morphogenesis).

Optimal growth in the extrauterine environment is expected to have beneficial outcomes for renal development. As discussed earlier, EUGR appears to influence renal function in preterm children[96] and, importantly, EUGR has been linked to early protein-caloric deficiency.[159] Therefore, it is important to optimize postnatal nutrition for preterm neonates in order to prevent further impairment of nephrogenesis in the extrauterine environment. To date, however, there is little understanding as to the nutrition required postnatally in order to parallel intrauterine growth in the preterm infant, hence further studies in this area are essential.

Conclusions

Although the consequences of preterm birth on renal function in the neonate have been well explored, the causes of renal

impairment and glomerular abnormalities in the kidneys of preterm infants are currently unknown. More efficient and accurate assessment of renal function in the clinical setting is necessary in order to identify neonates at risk of renal injury. Studies in the baboon model have provided important evidence that nephrogenesis unequivocally occurs postnatally. Alarmingly, however, many preterm baboon and human neonates exhibited abnormal glomeruli in the outer renal cortex, perhaps associated with factors in their postnatal clinical care. The challenge for the future, therefore, is to identify why nephrons formed in the extrauterine environment are vulnerable to abnormal development and to identify ways of minimizing glomerular injury. This knowledge is essential to maximize the number of functional nephrons at the beginning of life in preterm infants and consequently improve their long-term renal health and blood pressure regulation.

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REVIEW 2:

EFFECTS OF PRETERM BIRTH ON THE KIDNEY

CHAPTER ONE (REVIEW 2) DECLARATION

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

Nature of contribution	Extent of contribution (%)
Wrote 'renal function' section of manuscript	35%

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution (%) student co-authors only
Lina Gubhaju	Wrote some sections	35%
M. Jane Black	Wrote some sections	30%

Candidate's Signature [redacted] **Date** 30/04/12

Declaration by co-authors
The undersigned hereby certify that:

(7) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(8) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(9) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(10) there are no other authors of the publication according to these criteria;
(11) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(12) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) **Department of Anatomy & Developmental Biology, Monash University**

	Signature	Date
Lina Gubhaju	[redacted]	24/4/2012
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3

Effects of Preterm Birth on the Kidney

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1. Introduction

Preterm birth is the leading cause of morbidity and mortality in the neonatal period (Ward and Beachy 2003) and in childhood overall (McCormick, 1985). Over recent decades both the incidence of preterm birth and survival rates of preterm infants has increased, with babies born as early as 25 weeks gestation now having about an 80% chance of survival (Kutz et al., 2009). Preterm birth is defined as birth prior to 37 weeks of gestation; it can be further sub-classified as moderately preterm (birth between 32 and 37 weeks gestation) very preterm (birth < 32 weeks gestation) and extremely preterm (birth at < 28 weeks gestation) (Tucker and McGuire, 2004).

Due to the immaturity of the organs at the time of birth, preterm infants exhibit an increased risk of developing a number of postnatal complications including renal insufficiency and in severe cases renal failure (Drukker and Guignard, 2002; Choker and Gouyon, 2004); the mortality rate in these infants is very high (Drukker and Guignard, 2002; Andreoli, 2004). There is also evidence that preterm birth adversely affects nephrogenesis (the formation of nephrons) in the developing kidney; if this is the case, this has the potential to not only adversely affect renal function in the early postnatal period but to also increase the risk of renal disease later in life. Certainly, there are many studies linking a reduced nephron endowment early in life with hypertension (Keller et al., 2003; Luyckx and Brenner, 2005) and vulnerability to secondary renal insults in adulthood (Nenov et al., 2000; Zimanyi et al., 2006; Hoppe et al., 2007). In this regard, there is substantial recent epidemiological evidence linking preterm birth with an increase in blood pressure in adulthood (Siewert-Delle and Ljungman, 1998; Kistner et al., 2000; Kistner et al., 2002; Doyle et al., 2003; Bonamy et al., 2005; Hack et al., 2005; Johansson et al., 2005; Dalziel et al., 2007; Cooper et al., 2008; Keijzer-Veen et al., 2010b); these observations may be due to a reduced nephron endowment in preterm individuals.

In this chapter, we review the current knowledge of the effects of preterm birth on nephrogenesis in the developing kidney and on renal function postnatally.

2. The effects of preterm birth on nephrogenesis

The human kidney develops from a ridge of mesodermal tissue (known as the nephrogenic cord) which is found along the posterior wall of the abdominal cavity on either side of the primitive aorta (Blackburn, 2003). Development of the permanent kidney involves the formation of the pronephros and mesonephros (transitory organs) and the metanephros (the permanent kidney) (Saxen, 1987; Clark and Bertram, 1999; Sweeney and Avner, 2004; Moritz

et al., 2008). Development of the metanephros, begins at approximately week 5 of gestation with the outgrowth of the ureteric bud from the Wolffian duct (Saxen, 1987). Subsequent events include invasion of the mass of metanephric mesenchyme by the ureteric bud, followed by reciprocal inductive interactions between the ureteric bud and metanephric mesenchyme that lead to both dichotomous branching of the ureteric bud and the formation of nephrons at the ureteric bud tips (Moritz et al., 2008). Formation of the functional units of the kidney, the nephron, commences at approximately week 9 of gestation (Figure 1)(Blackburn, 2003).

As shown in the timeline in Figure 1, nephrogenesis in the human kidney is not complete until ~34-36 weeks of gestation with the majority of nephrons formed during the third trimester (from ~20 weeks of gestation onwards) (Hinchliffe et al., 1991). In very preterm and extremely preterm neonates, nephrogenesis is still on-going at the time of birth and continues in the *ex-utero* environment. Hence it is imperative to get a good understanding of how preterm birth affects the developing kidney and in particular the effects on nephrogenesis.

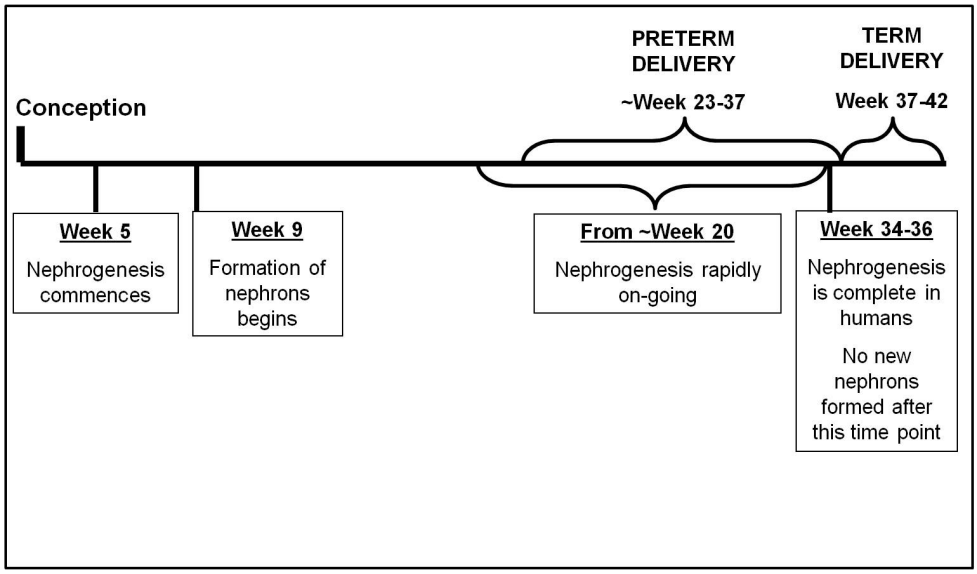

Fig. 1. A timeline of human nephrogenesis during gestation. Nephrogenesis is rapidly on-going at the time when most preterm neonates are delivered.

To date, there have been few studies examining the effects of preterm birth on nephrogenesis. *In vivo,* clinical studies have utilised renal ultrasound and magnetic resonance imaging (MRI) to estimate kidney size as a proxy measure of nephron endowment. However, such extrapolations should be treated with caution. Although kidney size is generally a good predictor of nephron number, this may not be the case in the preterm infant, with kidney size likely to be influenced by glomerular and tubular hypertrophy and increased interstitial mass due to the increased postnatal functional demands. Hence, it is often difficult to make predictions based on parameters such as kidney size (Lodrup et al., 2008). In this regard, autopsy studies in deceased preterm

neonates have provided insight into how preterm birth affects the structure of the kidney and the number of glomerular generations formed within the kidney. As well, carefully controlled experimental studies in the nonhuman primate provide valuable insight into the effects of preterm birth on nephrogenesis and on the total number of nephrons formed.

2.1 Clinical *in vivo* studies

Table 1 summarizes the main *in vivo* clinical studies that have investigated the effects of preterm birth on kidney length and volume. Overall, the findings in relation to the effects of preterm birth on kidney size are conflicting; however, it is difficult to compare between studies due to the varying time points of assessment and the differences in the control groups used in each study.

In order to establish the normal expected renal growth in preterm neonates, in a recent study van Venrooji *et al* (2010) examined kidney lengths and volumes of 30 very preterm neonates (gestational age ranging from 23.6 weeks to 30.6 weeks) at 1, 4 and 8 weeks after birth through ultrasound measurements. Significant correlations were found between average renal size (volume and length) with both body weight and age. The study also found no significant difference in growth rates between the extremely low birth weight group (<1.0 kg; GA 23.6 – 27.1 weeks) and the very low birth weight group (1.0 – 1.5 kg; GA 26.1 – 30.6 weeks). In another study, Huang and colleagues (2007) compared postnatal kidney growth in preterm neonates (<34 weeks gestation) to kidney growth *in utero* in a control group (28-40 weeks of gestation). Kidney volumes were measured in 56 preterm neonates at postnatal time points ranging from 14 to 96 days after birth. In the control group, kidney volumes were measured within 48 hours of birth. Kidney volumes of preterm neonates with a postconceptional age (defined as the sum of gestational age and postnatal age) equivalent to less than 31 weeks of gestation were significantly larger compared to controls, whereas the preterm neonates with a postconceptional age greater than 31 weeks of gestation had a significantly smaller kidney volume compared to controls. In the preterm infants where kidney volume increased, it is likely that the increase in kidney size is indicative of the response of the neonatal kidney to increased functional demands. However, if this is the case, it is unclear why the renal response was different in the preterm neonates greater than 31 weeks of postconceptional age where kidney volume was significantly less than controls. Another study by Kent *et al* (2009) compared MRI measurements of kidney volume and kidney volume relative to body weight in extremely preterm neonates (25-28 weeks gestation) to term-born controls. Kidney volumes in the preterm neonates were measured once they reached term-corrected age (37-40 weeks gestation) and within the first 4 weeks of life in the term controls. Interestingly in that study, no differences were found between groups.

Findings from older preterm infants, children and adults have been more consistent across studies and generally demonstrate a decrease in kidney size (relative to body size) compared to term controls. Firstly, Schmidt *et al* (2005) reported significantly smaller relative kidney volumes in preterm infants (born at less than 37 weeks of gestation) at 3 months of term-corrected age compared to 3-month-old term infants. Furthermore, preterm children at 18 months of term-equivalent age had slimmer shaped kidneys compared to term-born infants perhaps suggestive of decreased glomerular generations. In the most comprehensive study of renal growth in preterm infants to date, (involving 466 infants from 3 months through to two years of age), Drougia *et al* (2009) showed that kidney length was

Author & Year	Gestational age at birth (weeks), range	Age at assessment	Study Groups and sample size	Outcomes on renal size
Huang (2007)	24-36	14-96 days	Preterm (n=56) Gestational controls (n=44)	Larger relative volume in preterm <31 weeks PCA Smaller relative volume in preterm >31 weeks PCA
Van Venrooij (2010)	23-30	1, 4, 8 weeks	ELBW (n=14) VLBW (n=16)	No differences in volume or length
Kent (2009)	25-28	37-40 weeks (term equivalent)	Preterm (n=17) Term (n=13)	No differences in volume
Drougia (2008)	28-41	36 and 40 weeks (term equivalent) 3, 6, 12 and 24 months	Preterm 28-34 weeks (SGA: n=100 AGA: n=54) Preterm 34-36 weeks (SGA: n=80 AGA: n=61) Term (AGA: n=90 SGA: n=81)	Smaller right kidney length in preterm SGA 28-34 week group compared to AGA group at all postnatal time points
Schmidt (2005)	<37	40 weeks 3 and 18 months	Preterm (n=59) Term (n=801)	Smaller relative volumes at 3 and 18 months
Zaffanello (2010)	26-31	5-6 years	ELBW (n=36) VLBW (n=43)	ELBW have smaller volumes (Right, Left, Total) ELBW have smaller length (Right and Left)
Kwinta (2011)	26-29	6-7 years	ELBW (n=78) Term (n=38)	ELBW have smaller volume
Rakow (2008)	<32 (mean=27)	9-12 years	Preterm (n=33) Term (n=37)	Smaller absolute volume; not significant when adjusted for body surface area
Rodriguez-Soriano (2005)	23-35	6-12 years	Preterm (n=27)	Length and volume appeared to be in normal range; no comparison group
Keijzer-Veen (2010)	<32 (mean=31)	20 years	Preterm SGA (n=22) Preterm AGA (n=29) Term AGA (n=30)	Smaller length and volume in preterm (SGA and AGA) female group compared to term

Table 1. The main studies that have examined renal size (volume and length) in preterm neonates, children and adults (PCA=post-conceptional age, ELBW=extremely low birth weight, VLBW=very low birth weight, SGA=small for gestational age, AGA=appropriately grown for gestational age)

significantly decreased in small-for-gestational age preterm infants (born 28-34 weeks of gestation) compared to those born at term. In addition, Kwinta *et al* (2011) recently reported in 6 to 7 year old children, reduced absolute and relative kidney volumes in those born extremely low birth weight (26 – 29 weeks of gestation) compared to children born full-term. Furthermore, extremely low birth weight (birth weight <1.0kg; 26.3 - 27.7 weeks of gestation) children in a similar age group (5-6 year olds), had significantly reduced right and left kidney volumes and lengths compared to very low birth weight children (birth weight 1.0-1.5 kg; 29.9 - 31.3 weeks of gestation) (Zaffanello et al., 2010). This is the only study to date demonstrating significant differences in kidney size due to severity of prematurity.

To our knowledge, there has only been one study to date examining kidney size in preterm individuals in adulthood. In that study, 20-year-old adults born preterm (less than 32 weeks of gestation) had significantly smaller absolute and relative left kidney lengths and volumes compared to 20 year-old term controls; the difference was only significant in females (Keijzer-Veen et al., 2010a).

2.2 Human autopsy studies

There have been three published human autopsy studies (apart from case studies) that have examined the effect of preterm birth on postnatal nephrogenesis (Rodriguez et al., 2004; Faa et al., 2010; Sutherland et al., 2011b). Since non-uniform portions of the kidney are usually collected at autopsy, stereological methods cannot be accurately employed. Under these circumstances, the medullary ray glomerular generation counting method (Hinchliffe et al., 1992b) (also referred to as radial glomerular count or glomerular generation count) is a useful technique to provide insight into renal maturity and potentially nephron endowment. The method involves counting all developed glomeruli along one side of clearly distinguishable medullary rays in histological renal sections. Glomeruli are counted from the inner to outer renal cortex. Importantly, in our studies we have found a strong correlation between glomerular generation number and nephron number, which supports the validity of the technique (Sutherland et al., 2011a).

In one of the first autopsy studies conducted, the number of radial glomerular counts in kidneys from extremely preterm neonates (56 neonates) was compared to 10 full-term infants (Rodriguez et al., 2004). Radial glomerular counts were found to be significantly reduced in preterm infants; however, since many of the preterm infants were also intrauterine growth restricted (IUGR) it is difficult to determine the effects of preterm birth *per se* from this study. In a smaller study, Faa *et al* (2010) have reported significantly reduced radial glomerular counts and marked inter-individual variability in the number of glomerular generations among the kidneys from preterm neonates compared to term newborns. In that study, 8 human fetuses, 12 preterm neonates and 3 full-term neonates were examined; it is unknown whether any of the neonates were also IUGR.

As follow on to these studies, we have recently undertaken a study examining kidneys obtained at autopsy from 28 preterm infants and 32 still-born gestational controls (Sutherland et al., 2011b); the preterm group included 6 infants that were also IUGR. Importantly, analyses comparing growth restricted and non-growth restricted kidneys demonstrated no significant differences, although the findings are limited by the small sample of growth restricted neonates. In contrast to the studies described above, we found accelerated nephrogenesis in the preterm group demonstrated by an increase in the number of glomerular generations, a decreased nephrogenic zone width (suggesting

earlier cessation of nephrogenesis postnatally) and a decreased proportion of glomeruli in the immature V (vesicle) -stage of maturation compared to still-born gestational controls. Furthermore, mean renal corpuscle cross sectional area was significantly larger in the preterm kidneys. Of particular concern, kidneys from preterm infants had a higher percentage of structurally abnormal glomeruli compared to the gestational controls with up to 13.7% of glomeruli affected. These abnormal glomeruli exhibited a dilated Bowman's space and shrunken glomerular tuft. The factors associated with the development of abnormal glomeruli are yet unknown and this is an important area of future research.

2.3 Nonhuman primate animal studies

We have shown that the baboon is an ideal model to study human kidney development, as the ontogeny of the kidney very closely matches that of the human (Gubhaju and Black, 2005). Similar to the human, nephrogenesis in the baboon commences at approximately 30 days of gestation (Hendrickx et al., 1971) and ceases prior to term by 175 days gestation (Term = 185 days gestation) (Gubhaju and Black, 2005). Similar to the wide range in nephron number found in human kidneys, in the kidneys we examined total nephron number ranged from 193,983 to 334,316 in baboons delivered at term.

In collaboration with researchers at the Southwest Foundation for Biomedical Research (San Antonio, Texas, U.S.A) we have examined the kidneys from fetal baboons that have been prematurely delivered and ventilated after birth in a neonatal intensive care unit (NICU) in a similar manner to human preterm babies (Gubhaju et al., 2009). These appropriate weight-for-gestational age baboons were delivered extremely preterm (125 days of gestation); equivalent to approximately 27 weeks gestation in humans. After birth, all preterm neonates were intubated, administered 100 mg/kg surfactant (Survanta; donated by Ross Products, Columbus, OH), and ventilated with pressure limited infant ventilators (InfantStar; donated by Infrasonics, San Diego, CA). All preterm neonates were also treated with ampicillin and gentamicin for the first 7–10 days of life (Thomson et al., 2004). Further doses of antibiotics were only administered in cases of clinically suspected infection. Following birth, the baboon neonates were ventilated in the NICU for a maximum period of 21 days.

In this model, kidney volume, nephron number and size of the renal corpuscle were estimated using unbiased stereology, the gold standard method for the determination of nephron number (Bertram, 2001; Sutherland et al., 2011a). One of the most significant findings from the nonhuman primate studies was the clear evidence that nephrogenesis was on-going in the extrauterine environment following preterm birth. There was structural evidence of on-going nephrogenesis in the outer renal cortex (branching of the ureteric bud, metanephric mesenchyme and Comma and S-shaped bodies) and this was accompanied by a significant increase in the number of glomerular generations and nephron number in the postnatal environment by postnatal day 21 (Gubhaju et al., 2009). Furthermore, kidney weight and volume relative to body weight were significantly higher in the preterm baboon neonates compared to gestational age-matched controls; a finding that has been previously reported in human studies (Huang et al., 2007). There was a significant decrease in glomerular density (glomeruli/gram of kidney) in the kidney from preterm baboon neonates compared to gestational controls suggestive of altered renal growth and potentially an increase in tubular mass.

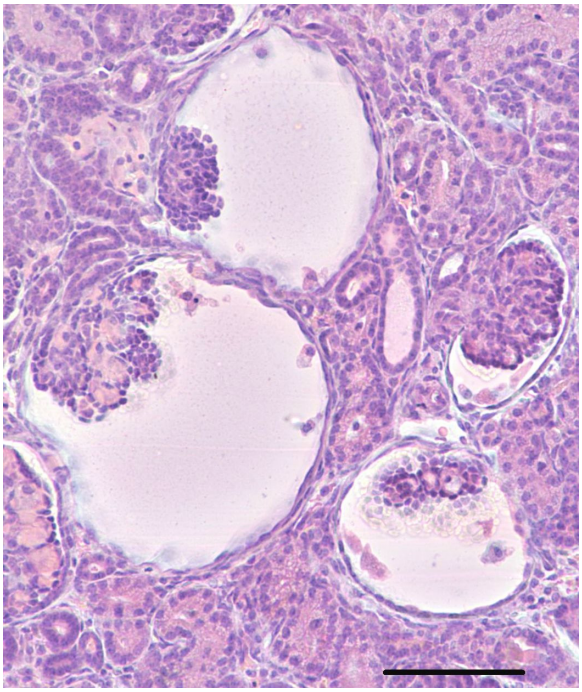

Fig. 2. A representative photomicrograph of a histological renal section from a preterm baboon showing abnormal glomeruli in the outer renal cortex; these morphologically immature glomeruli exhibited a shrunken glomerular tuft and enlarged Bowman's space. Scale bar = 100 μm

Similar to the findings from the human autopsy studies, morphologically abnormal glomeruli were also found in kidneys from preterm baboon neonates; with up to 18% of glomeruli affected (Figure 2). High proportions of abnormal glomeruli were only found in those kidneys from preterm baboons, whereas in gestational controls the proportion of abnormal glomeruli was negligible. The observed abnormal glomeruli were only present in the superficial outer cortex of the preterm kidney suggesting that it is the glomeruli that are recently formed (possibly those formed in the extrauterine environment) that are 'at risk.' Further immunohistochemical analyses demonstrated that the abnormal glomeruli were poorly vascularised (lack of endothelial cell marker, CD31 immunostaining). In addition, immunostaining with the podocyte marker, WT-1, revealed that the abnormal glomeruli were in a relatively immature stage of development since the glomerular tuft contained WT-1 positive cells surrounding a mass of relatively undifferentiated cells. Importantly, there were a large number of parietal epithelial cells surrounding the Bowman's capsule; previous human studies have reported a similar morphology in atubular glomeruli (Gibson et al., 1996; Bariety et al., 2006). If the abnormal glomeruli in the preterm kidneys are atubular, then they will never be functional. Further studies are required to determine whether this is the case. Certainly, a large proportion of non-functional glomeruli in the preterm kidney is likely to have adverse consequences on renal function both in the neonatal period and in the long-term (by reducing the functional reserve of nephrons).

3. Renal function in the preterm neonate

There have been a number of studies that have examined the effects of preterm birth on renal function. However, it must be kept in mind when interpreting the data from these studies that the function of the immature preterm kidney is likely to be quite different to that of the term infant, which in turn is likely to be quite different to the adult. Hence, although the 'normal' levels of the standard markers of renal function (such as serum creatinine and urinary albumin) have been well-established for the adult population, the standard levels in the neonate, especially those of the preterm neonate, are not clearly defined. This often makes the clinical assessment of renal function in the preterm neonate difficult. In future research, it is necessary to establish the 'normal' levels of renal function in the preterm infant and to identify robust biomarkers for the early diagnosis of renal injury in the neonatal period, which may in turn prevent long-term renal dysfunction.

3.1 Fluid and electrolyte homeostasis

An imbalance of fluid and electrolyte intake versus excretion is very common in premature neonates, and can lead to significant morbidity and mortality (Bhatia, 2006); hypernatraemia, for example, can result in severe neurological injury (Moritz and Ayus, 2005). Insensible fluid loss is a major factor (Bhatia, 2006), and is primarily transcutaneous due to the developmental immaturity of the skin and a high body surface area to body water mass ratio (Baumgart and Costarino, 2000). Equally, the delayed loss of extracellular fluid volume following preterm birth is also associated with an increased risk of morbidity, in particular bronchopulmonary dysplasia (Oh et al., 2005) and patent ductus arteriosus (Bell and Acarregui, 2008).

Three phases of fluid and electrolyte homeostasis have been observed in the immediate period following preterm birth; these phases occurred similarly in extremely low birth weight infants and those at older gestational ages (Lorenz et al., 1982; Lorenz et al., 1995). As described by Lorenz *et al.*, (1982; 1995) in the first 24 hours following birth, a period known as the pre-diuretic stage, urine output is minimal and sodium excretion is low. On postnatal days 2-3, termed the diuretic phase, sodium excretion and urine output significantly increase, which occurs independently of fluid intake. From approximately days 4-5 of life, the post-diuretic phase, urine output changes in response to fluid intake (Lorenz et al., 1982; Lorenz et al., 1995). Importantly, however, the postnatal time-point that these phases occur, and their duration, differ between individual neonates (Lorenz et al., 1995), as does the amount of insensible fluid loss; together, this highlights the need for an individualised approach to fluid therapy in preterm neonates.

Urine output is the most commonly and easily measured indicator of renal function in the preterm neonate. Urine output less than 0.5 ml/kg/h, known as oliguria, can be indicative of acute kidney injury (AKI). AKI, however, can also be non-oliguric, therefore urine output is not a very specific indicator of renal function. Furthermore, from the post-diuretic phase of fluid homeostasis urine output is highly dependent upon fluid intake; high intakes may artificially increase urine output, while not accurately reflecting renal functional capacity.

The most common measure of electrolyte balance in the neonate is the calculation of the fractional excretion of sodium (FENa), which is the percentage of sodium that is excreted and not taken up through tubular reabsorption. The calculation of FENa takes into account the levels of both serum and urine sodium, and it is corrected for serum and urine creatinine levels. Therefore, high urine sodium levels may be indicative of structural immaturity of the

renal tubule (short length of the tubules, and changes in the density and structure of transporter proteins) (Jones and Chesney, 1992), or due to renal injury (Ueda and Shah, 2000; Bonventre, 2007).

Studies that have assessed FENa during the neonatal period have determined that sodium excretion is significantly higher in preterm neonates compared to term controls (Siegel and Oh, 1976; Aperia et al., 1981), and significantly decreases with increasing gestational (Gallini et al., 2000) and postnatal age (Ross et al., 1977; Sulyok et al., 1979; Aperia et al., 1981; Gallini et al., 2000; Giapros et al., 2007). Therefore, with increasing renal maturity a positive sodium balance (low FENa) is achieved, which is essential for the growth and development of the neonate and the maintenance of fluid homeostasis (Engle, 1986).

3.2 Glomerular filtration rate

Endogenous creatinine is the most practical and commonly used marker of renal function, with calculated creatinine clearance widely used as an estimate of glomerular filtration rate (GFR). In the clinical setting, repeated serum creatinine levels are used to gauge renal function in neonates; this is an easily obtainable measure via routine blood collection and does not rely on timed urine samples or additional invasive procedures. This method does, however, have significant limitations. Immediately following birth, serum creatinine levels are equivalent to the fetal levels, which during the third trimester of gestation rise from 42 μmol/L at 23 weeks to 47 μmol/L at term; the increase likely reflecting an increase in muscle mass (Moniz et al., 1985). In the first forty-eight hours following birth, however, serum creatinine levels significantly increase (Bueva and Guignard, 1994; Miall et al., 1999). This is considered to be due, in part, to tubular creatinine reabsorption, as has been evidenced in a neonatal animal model (Matos et al., 1998), and also due to the inadequacy of glomerular filtration during the early postnatal period (Miall et al., 1999). Peak serum creatinine levels are reached at postnatal day 2-4 of life, with the highest levels and most delayed timing of the peak creatinine level seen in neonates at the lowest gestational ages (Miall et al., 1999).

During the first week of life following preterm birth, GFR is significantly lower in preterm neonates than in term-born controls (Siegel and Oh, 1976; Finney et al., 2000; Schreuder et al., 2009), and is significantly positively correlated with both gestational age at birth, and postnatal age (Clark et al., 1989; Gordjani et al., 1998; Iacobelli et al., 2009). Compared to term neonates, the rate of increase in GFR after birth is slower in neonates born preterm (Gordjani et al., 1998). Up until two months of age there are similar findings, with a number of studies observing an increase in GFR concurrent to increasing gestational and postnatal ages (Ross et al., 1977; Fawer et al., 1979; Sulyok et al., 1979; Aperia et al., 1981; Wilkins, 1992; Bueva and Guignard, 1994; Gallini et al., 2000; Cuzzolin et al., 2006; Thayyil et al., 2008). Although a number of studies have now been performed in this area, there is still a lack of clear definition regarding expected GFR values in the preterm neonate. Recently published standard curves of GFRs in neonates born at 27-31 weeks gestational age, from 7 to 28 days of life, will go some way in aiding in the clinical interpretation of renal function in this particular group of neonates (Vieux et al., 2010).

Given that age has been found to be a strong determinant of GFR, the low GFR observed in the preterm neonate after birth is likely the result of renal immaturity (a low number of filtering glomeruli), and it is also likely to be influenced by differences in renal blood flow and vascular resistance. It is essential that GFR is monitored in the postnatal period following preterm birth, as a very low GFR is likely to impair renal drug clearance, leading to nephrotoxicity.

3.3 Acute kidney injury

Acute kidney injury (AKI; previously referred to as acute renal failure) is reported to occur in 8% to 24% of preterm neonates admitted to neonatal intensive care units (Stapleton et al., 1987; Hentschel et al., 1996). The current diagnosis of AKI is primarily based on the RIFLE system, which categorises the stages of increasing AKI severity: Risk, Injury, Failure, Loss and End-stage kidney disease (ESKD) (Bellomo et al., 2004). This system was further modified following recommendations from the acute kidney injury network (AKIN) (Mehta et al., 2007). The initial clinical indication of AKI risk includes a 50% increase in serum creatinine (or ≥ 0.3 mg/dl within a 48 hour period), and/or a urine output less than 0.5 mg/kg/hr for a period of six hours (Bellomo et al., 2004; Mehta et al., 2007), which are changes indicative of a significantly reduced GFR. Classifications for the definition of AKI in a neonatal specific population, however, have not been developed.

The causes of AKI in the preterm neonate are primarily pre-renal in origin, arising from conditions which affect renal perfusion such as hypotension, hypoxia and sepsis (Stapleton et al., 1987; Cataldi et al., 2005). These in turn lead to apoptotic, necrotic and inflammatory processes within the kidney (Ueda and Shah, 2000; Bonventre, 2007). Importantly, AKI in the preterm neonate may subsequently lead to long-term chronic renal disease (Abitbol et al., 2003).

In a study involving 172 preterm neonates by Cataldi *et al*. 2005, the risk factors for AKI were found to be maternal and neonatal drug administration (non-steroidal anti-inflammatory drugs (NSAIDs) and antibiotics, especially ceftazidime), a low Apgar score, and a patent ductus arteriosus. Interestingly, gestational age did not affect risk of AKI, however, the majority of AKI cases (79%) weighed < 1.5 kg at birth (Cataldi et al., 2005). In a larger study by Cuzzolin *et al*. (2006), involving 281 preterm neonates, a number of risk factors for AKI were also identified. These included maternal NSAID administration, low Apgar score, respiratory distress syndrome, neonatal drug administration (antibiotics and NSAIDs), and a number of clinical interventions (intubation at birth, catheterization, phototherapy, and mechanical ventilation).

Given the importance of the early diagnosis and treatment of AKI, there has been much recent focus on the discovery of novel urinary biomarkers. The expectation of a new biomarker is to enable the diagnosis of cellular injury before a decline in renal function occurs. For example, serum creatinine is not elevated until 48-72 hours after an acute injury has occurred (Moran and Myers, 1985); such a prolonged delay before diagnosis and treatment likely results in further renal injury. As Rosner (2009) describes, it would be optimal if a biomarker could be developed to: 1) assess the response to, and any adverse effects of therapeutic interventions 2) indicate the severity of renal injury 3) inform on the etiology of the injury and 4) identify the location of injured cells. In a systematic review of the current literature, Parikh *et al*. (2010) determined that the molecules with the most promise for the diagnosis of established AKI include interleukin-18 (IL-18), kidney injury molecule-1 (KIM-1), N-acetyl-beta-D-glucosaminidase (NAG) and neutrophil gelatinase-associated lipocalin (NGAL). NGAL, IL-18, fatty acid binding protein (FABP), and cystatin-C are the most encouraging biomarkers for the early diagnosis of AKI, given that the upregulation of these molecules following injury onset precedes the rise in serum creatinine by many hours (Parikh et al., 2010).

In the preterm neonate, a small number of studies have been conducted for the assessment of urinary NGAL levels, with mixed results. These studies have shown that the highest NGAL levels are evident in the neonates that are critically ill, with and without evidence of

renal dysfunction (Lavery et al., 2008; Parravicini, 2010); in particular, NGAL shows potential as a promising biomarker of late-onset sepsis (Lavery et al., 2008; Parravicini et al., 2010). Urinary NGAL levels also strongly correlated with gestational and postnatal age (Lavery et al., 2008; Huynh et al., 2009), perhaps reflecting the renal production of NGAL during nephrogenesis (Gwira et al., 2005) which is often still ongoing during the early postnatal period. Normative values for urinary NGAL in preterm neonates with uncomplicated clinical courses have also been published, with the results indicating a greater variation in females than males (Huynh et al., 2009).

3.4 Proteinuria

Proteinuria, the presence of high levels of protein in the urine, may be of glomerular and/or tubular origin. The number of different proteins that have been identified in the adult urinary proteome is 1,543, and these are primarily of membrane, extracellular and lysosomal origin (Adachi et al., 2006). Despite this large number, unless renal function is impaired, proteins are normally only present at very low levels in urine, due to the function of the glomerular filtration barrier and tubular reabsorption capabilities.

Presence of high molecular weight (HMW) proteins in the urine, such as albumin traditionally indicates a disruption in the integrity of the glomerular filtration barrier. Recent debate, however, has suggested that the contribution of tubular reabsorption of albumin from the filtrate may be greater than previously considered (Comper et al., 2008). In general, albuminuria is a strong marker for renal and cardiovascular disease, and a risk factor for mortality (Matsushita et al., 2010; Methven et al., 2011). Normally, adults excrete less than 30 mg of albumin per 24 hours (Mathieson, 2004). Urinary albumin levels between 30 – 300 mg in 24 hours is considered microalbuminuria, with levels greater than 300 mg classified as macroalbuminuria (Mathieson, 2004). Traditionally, 24 hour urine samples were required for reliable estimates of urinary protein. However, single random spot samples with protein levels corrected for urine creatinine, have been shown to be significantly correlated with results from 24 hour collections, and are equally effective in the prediction of outcomes (Ralston et al., 1988; Methven et al., 2011). In neonates, 24 hour urine collection is difficult, therefore analysis of urinary protein levels are undertaken using spot urine samples obtained using urine collection bags.

Low molecular weight (LMW) proteins, such as α1-microglobulin, β2-microglobulin and retinol binding protein pass freely through the glomerular filter and undergo reuptake via proximal tubule cells (Tomlinson, 1992). Megalin and cubulin have been identified as important receptors involved in tubular protein uptake, with mutations in the receptors resulting in proteinuria (Christensen and Birn, 2001). To date, LMW protein levels in the urine are not routinely measured in the clinical setting. Importantly, however, amongst the LMW proteins there may be potential novel biomarkers of tubular cell injury and this requires further research (Rosner, 2009; Parikh et al., 2010).

In the preterm neonate, few studies have been conducted to examine urine protein excretion. In general, there is a high variability in urine albumin levels between individual neonates (Clark et al., 1989; Fell et al., 1997), with the highest levels exhibited by those with a low gestational age at birth and those that are clinically unstable (Galaske, 1986; Clark et al., 1989; Tsukahara et al., 1994; Fell et al., 1997; Awad et al., 2002b). The majority of studies have only been conducted during the first week of life following preterm birth. However, in a study by Tsukahara *et al.* (1994) urine albumin levels were assessed in preterm and term

neonates over the first 28 days of life. Urine albumin levels were found to remain relatively stable postnatally over the one month period in the term neonates, whereas in the preterm neonates, urine albumin was seen to decrease with increasing postnatal age. These findings suggest that the glomerular filtration barrier following preterm birth is structurally immature, until beyond one month of age.

Urinary β2-microglobulin levels have also been shown to be significantly greater in the preterm infant compared to term-born infants throughout the first month of life (Aperia et al., 1981; Tsukahara et al., 1990; Tsukahara et al., 1994), and are decreased with increasing gestational and postnatal age (Takieddine et al., 1983). Similarly, levels of α1-microglobulin and RBP are higher in preterm neonates than neonates born at term (Clark et al., 1989; Fell et al., 1997; Awad et al., 2002a). To date, however, it remains unclear whether the increased urinary high- and low- molecular weight protein levels reported in preterm neonates are associated with renal immaturity and/or injury. The high variability in urinary protein levels may also reflect differences in the postnatal clinical course in preterm neonates; further studies are necessary to verify whether this is the case.

3.5 Long-term effects of preterm birth on renal function

Renal function in preterm-born children and adults, has to date only been investigated in a small number of studies, with inconclusive results. In school-aged children, Rakow *et al.* (2008) found no difference in GFR or urinary levels of both HMW and LMW proteins between children born less than 32 weeks gestational age, and those that were born at term. Similarly, Vanpee *et al.* (1992) determined no difference in renal function in preterm and term-born children at 8 years of age, despite lower GFR and higher urine albumin levels being evident in the preterm group at 9 months of age. In contrast, however, a study by Rodriguez-Soriano and colleagues (2005) reported that GFR was significantly reduced in preterm-born children compared to term controls, with impairments in electrolyte excretion also evident. Furthermore, in children examined at 6-8 years of age, Iacobelli et al. (2007) demonstrated microalbuminuria in 8.3% of the preterm neonates, which was associated with postnatal factors such as neonatal hypotension and increased catch-up growth. Increased risk of renal demise was also evident in individuals born preterm who were obese during childhood (Abitbol et al., 2009).

Two studies have also been conducted to examine renal function in young adults (20-30 years of age), with both Keijzer-Veen *et al.* (2007) and Kistner *et al.* (Kistner et al., 2000) finding no effect of preterm birth on GFR or albuminuria. To the contrary, in a cohort of 19 year old young adults, those who were born preterm as well as IUGR, there was a significant reduction in GFR (Keijzer-Veen et al., 2005). Given these results in preterm-born children and adults, there is some suggestion that preterm birth adversely affects the growth and functional capacity of the kidney and may result in progressive renal failure later in life. Importantly, adverse consequences appear to be more likely to occur in combination with other insults. Therefore, future research must be directed towards identifying these insults and their effects on the structure and function of the kidney.

4. Preterm birth leads to glomerular abnormalities – Areas of future research

One of the most important findings we have shown thus far, is the presence of abnormal glomeruli in both the human and nonhuman primate (baboon) preterm kidney. These abnormal glomeruli are located in the outer renal cortex and are in the most immature stage of development (stage 1); they are composed of an undifferentiated glomerular anlage of cells

(foundation group of cells) surrounded by a layer of podocytes with scant, if any, capillarisation. Our findings thus strongly suggest that it is the very immature glomeruli (possibly those formed in the extrauterine environment) that are particularly vulnerable to preterm birth. Given the gross abnormalities observed in these glomeruli, it is unlikely that these glomeruli will ever be functional and thus, it is expected that they will be subsequently resorbed into the surrounding tissue. In the short-term, such abnormalities will likely lead to marked impairment of renal function in the neonate if a high proportion of the nephrons are affected, or to minor impairment if only a small proportion are abnormal. When the kidney is severely affected this will adversely impact on the number of functional nephrons at the beginning of life and thus reduce the long-term functional reserve of the kidney, rendering it vulnerable to hypertension and secondary life style insults.

The cause(s) of the glomerular abnormalities in the preterm infant is currently unknown. Importantly in this regard, we have shown that there is a wide variation in the proportion of abnormal glomeruli within the kidneys of preterm infants, with the kidneys of some preterm infants appearing morphologically normal whereas in others a large proportion of the glomeruli appear abnormal (Sutherland et al., 2011b). Given the wide variation in the proportion of abnormal glomeruli within the kidneys of preterm infants, this suggests that it is not preterm birth *per se* that leads to the glomerular abnormalities; instead they are likely due to factors often associated with preterm birth as shown in Figure 3. It is likely that these deleterious effects may relate to: 1) adverse factors in the *in utero* environment that have led to premature delivery, 2) factors in the neonatal care of the preterm infant and 3) pharmacological interventions/therapies administered to mothers prior to birth and/or the infant after birth. There are many factors which apply to each of these categories; below we have selected some that we consider are important for future research.

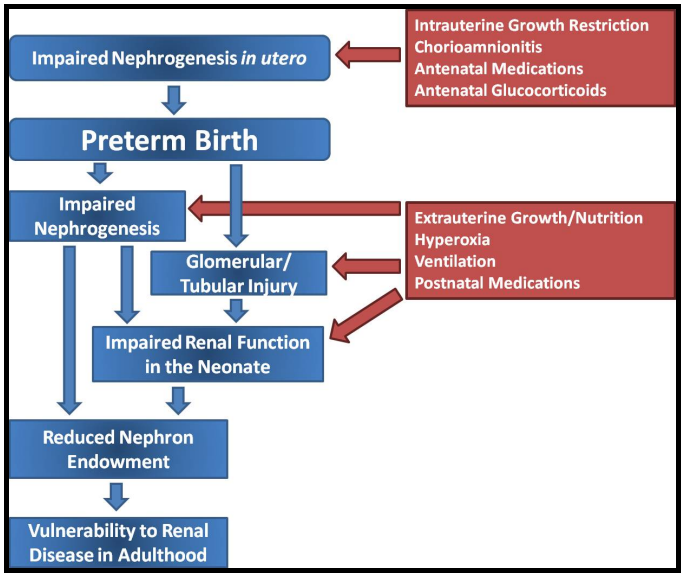

Fig. 3. Depiction of the potential factors that may contribute to impaired nephrogenesis in the preterm kidney and consequently lead to a reduced nephron endowment and vulnerability to renal disease in adulthood

4.1 Adverse factors in the *in utero* environment

Two potential factors in the *in utero* environment that may render the preterm kidney vulnerable are IUGR and/or exposure to chorioamnionitis.

4.1.1 Intrauterine growth restriction

IUGR (growth below the 10th percentile for gestational age) is often a co-morbidity of preterm birth. Certainly, it is well described in both human and experimental models that IUGR leads to a reduced nephron endowment at birth (Hinchliffe et al., 1992a; Merlet-Benichou et al., 1994; Manalich et al., 2000; Zimanyi et al., 2004; Zohdi et al., 2007). This is likely due to the reduced growth of the fetal kidney (with the number of nephrons directly proportional to kidney size). In this regard, there is often redistribution of blood flow in the growth-restricted fetus, leading to preferential blood flow to the brain (termed brain sparing) and reduced blood flow to organs such as the kidneys (Behrman et al., 1970; Gunnarsson et al., 1998). To our knowledge the impact of reduced blood flow to the formation of nephrons in the IUGR fetal kidney has not been investigated. Given the dramatic change in hemodynamics at the time of birth (elevation in blood pressure and increased renal blood flow) it is conceivable that the recently formed glomeruli in the IUGR kidney (with a reduced renal blood flow prenatally) may be particularly vulnerable to the haemodynamic transition at birth. This is an important area of research that needs to be thoroughly investigated.

4.1.2 Chorioamnionitis

Chorioamnionitis (a bacterial infection of the chorion and amnion) is a common antecedent of preterm birth (Romero et al., 2006; Goldenberg et al., 2008), especially in births prior to 30 weeks of gestation (Lahra and Jeffery, 2004); it is often complicated by IUGR. Chorioamnionitis may manifest as either a clinical or subclinical condition. When severe, it can ultimately give rise to the fetal inflammatory response syndrome which is characterised by funisitis, fetal vasculitis and an increase in of pro-inflammatory cytokines in fetal blood and amniotic fluid (Romero et al., 1998; Romero et al., 2003; Gotsch et al., 2007). It is likely that the fetal systemic inflammatory response will lead to renal inflammation in chorioamnionitis-exposed infants; conceivably if present *in utero* this will have deleterious effects on nephrogenesis, and if present at the time of delivery, it will adversely impact on renal function. In this regard, we have recent evidence in fetal sheep to demonstrate that exposure to chorioamnionitis late in gestation does adversely affect nephrogenesis, leading to a 23% and 18% reduction in nephron endowment in singleton and twin-exposed fetuses, respectively (Galinsky et al., 2011). It is important to note that we did not observe any morphological abnormalities in the glomeruli of these fetal kidneys. However, it is conceivable, that if renal inflammation is present at the time of birth, that this will further compromise postnatal nephrogenesis and renal function in the neonate. Following the hemodynamic transition at birth, it may be then that glomerular morphological abnormalities develop in these already compromised kidneys. Importantly in this regard, a multiple logistic regression analysis of 2508 preterm neonates, treated with indomethacin, showed a significant correlation between intrauterine inflammation and prevalence of renal and electrolyte abnormalities (Itabashi et al., 2003), thus suggesting that intrauterine inflammation in concert with postnatal indomethacin treatment can lead to renal dysfunction. Certainly our results support the idea that prematurity, when complicated with

chorioamnionitis, is likely to exacerbate postnatal renal dysfunction and further studies are required to determine whether renal inflammation at the time of birth is associated with the formation of abnormal glomeruli in the neonatal period.

4.2 Factors in the neonatal care of the infant

4.2.1 Hyperoxia

The administration of supplemental oxygen to preterm infants experiencing respiratory distress is standard therapy, however, the effects of high levels of oxygen in the bloodstream on the development of organs is not well understood. Although the levels of supplemental oxygen administered to the preterm infant have been substantially reduced in recent years, the levels of oxygen in the bloodstream remain elevated above normal; hence it is important that research is conducted into the effects of hyperoxia on nephrogenesis in the preterm infant. Certainly, experimental studies in the lung have shown that hyperoxia can lead to the generation of oxygen free radicals, infiltration of inflammatory cells, collagen deposition, cellular apoptosis and subsequent tissue injury (McGrath-Morrow and Stahl, 2001; Dieperink et al., 2006; Alejandre-Alcazar et al., 2007; Chen et al., 2007; Chetty et al., 2008). Whether this is also the case in other tissues has not been examined.

4.2.2 Ventilation

The mode of ventilation of the preterm infant also has the potential to impact on the developing kidney, since alterations in airway pressure are reported to lead to significant cardiopulmonary haemodynamic changes (Polglase et al., 2009) which may subsequently affect renal perfusion. For instance, mechanical ventilation has been shown to alter renal hemodynamics by leading to an increase in intrathoracic pressure, therefore decreasing cardiac output, leading to renal vasoconstriction and decreased GFR (Arant, 1987). It is likely that changes in renal blood flow will directly influence the growth of the kidney. Hence, it is important in future research to determine the effects of altered renal hemodynamics on growth of the developing kidney and how this is influenced by different modes of ventilation.

4.2.3 Extrauterine growth / nutrition

In general, postnatal growth of the preterm infant is markedly attenuated when compared to the term infant and when compared to the normal rate of growth *in utero* for the same post-conceptional age (Ehrenkranz, 2000; Clark et al., 2003). Extrauterine growth restriction in premature neonates (defined as growth below the 10th percentile of intrauterine growth expectation) is likely to directly influence nephrogenesis. In support of this concept, Bacchetta et al (2009) reported lower GFRs (albeit in the normal range) in 7 year old children who had been born very preterm (< 30 weeks gestation) and who were either IUGR or extrauterine growth restricted. However, it is important to note that in some preterm infants there can be a disproportional increase in kidney size (relative to body weight) after birth (Huang et al., 2007; Sutherland et al., 2011b) most probably due to the increased functional demands on the kidney. Importantly we have shown in our preterm baboon studies that under these circumstances, there remains a significant correlation between kidney size and nephron number (Gubhaju et al., 2009).

Taken together, these studies highlight the importance that neonatal nutrition can potentially have on kidney development in the preterm infant. Since nephrogenesis is

ongoing after birth in the preterm infant, this provides a window of opportunity whereby early postnatal nutrition in the intensive care unit may be able to directly influence the number of nephrons formed within the kidney. Hence optimising nutrition in the neonatal period, with an aim to maximising nephron endowment in the preterm newborn, is an important area for future research. At this stage, there is no known maternal nutrient supplementation that can improve renal outcomes in the fetus; it is critical to investigate this.

4.3 Pharmacological treatments to the mother prior to birth and / or the infant after birth

There are a number of medications routinely administered to women during pregnancy and to preterm infants. Some of the most commonly used are: 1) antenatal glucocorticoids which are routinely administered either to the mother 'at risk' of preterm delivery (Vidaeff et al., 2003) or to the preterm infant immediately after delivery to accelerate lung maturation in the infant, 2) antibiotics, often administered to the mother with chorioamnionitis and to the infant with postnatal conditions such as necrotising enterocolitis (Gortner et al., 1991) 3) non steroidal anti-inflammatory drugs (such as ibuprofen and indomethacin) which are often administered to close a patent ductus arteriosus (Ellison et al., 1983) and 4) inotropes (such as dopamine and dobutamine) which are administered in cases of hypotension and poor blood flow (Osborn et al., 2002). Importantly, and of concern, all these medications have the potential to adversely impact on nephrogenesis.

4.3.1 Glucocorticoids

In addition to leading to lung maturation in the preterm infant, administration of glucocorticoids has been favourably reported to accelerate renal maturation thus establishing an adequate GFR and efficient tubular reabsorption (Ervin et al., 1996; Ervin et al., 1998; Petershack et al., 1999). However, the question remains: 'Does this acceleration in renal maturation lead to an abnormal and rapid cessation of nephrogenesis which would ultimately affect nephron endowment? In support of this, a number of experimental studies (conducted in animal models) have shown that exposure of the fetus to maternal glucocorticoids can lead to reduced nephron endowment in the offspring (Celsi et al., 1998; Ortiz et al., 2001; Moritz et al., 2002). As follow up to these studies, in a carefully controlled study in our baboon model we have examined the effect of administration of antenatal betamethasone (intramuscular injection of 6mg at 48 hours and 24 hours prior to preterm delivery) on nephrogenesis in the neonatal baboon kidney (Gubhaju et al., 2009). We found that although there was acceleration of glomerular maturation in the betamethasone-treated baboons, the total number of nephrons was within the normal range and importantly we demonstrated that fetal exposure to maternal glucocorticoids was not the cause of the glomerular abnormalities associated with preterm birth.

4.3.2 Antibiotics

The use of antibiotics is often essential in the treatment of the mother during pregnancy and of the preterm infant after birth. Alarmingly, it has been shown that antibiotics, such as the aminoglycosides, can be nephrotoxic in the newborn with the preterm infant most vulnerable (Giapros et al., 2003). This is of concern, given that aminoglycosides are not metabolised in the body and thus accumulate in the kidney where they are eventually

eliminated (Nagai and Takano, 2004). In experimental studies antibiotics are also linked with impairment of nephrogenesis (Gilbert et al., 1990; Gilbert et al., 1994; Cullen et al., 2000; Nathanson et al., 2000). For instance, it has been shown that incubation of metanephroi in culture with gentamicin leads to decreased branching morphogenesis of the ureteric tree; this is a likely mediator of the reduction in the number of nephrons formed (Cullen et al., 2000). Given that antibiotics can readily cross the placenta, plus their wide use in the neonatal care of the preterm infant, it is imperative in future studies to gain a more precise understanding of the dose, duration and class of antibiotic treatment that leads to adverse effects in the neonatal kidney; such information would likely influence the care in the neonatal intensive care unit.

4.3.3 Non steroidal anti-inflammatory drugs

NSAIDs, such as ibuprofen and indomethacin are usually administered to preterm neonates to stimulate closure of a patent ductus arteriosus; this can occur in up to 80% of extremely preterm infants (Ellison et al., 1983). Importantly, there have been a number of studies that have reported adverse effects on both the structure and function of the preterm kidney following treatment with NSAIDs. For example, renal insufficiency, demonstrated by a significant increase in serum creatinine has been reported in infants following either antenatal or postnatal exposure to NSAIDs (Kang et al., 1999; Butler-O'Hara and D'Angio, 2002). Of concern, in a case-controlled study where renal impairment was reported in preterm neonates that had received indomethacin treatment for a patent ductus arteriosus, 24% of the babies suffered acute renal failure (Akima et al., 2004). In addition, in the rat model where, similar to the preterm infant, nephrogenesis is ongoing after birth, exposure to indomethacin, ibuprofen and gentamicin have all been shown to lead to renal injury in the immature kidneys. There was evidence of vacuolization of epithelium and loss of microvilli in proximal tubules, effacement of podocyte foot processes and irregularities of the basement membrane in the glomeruli and edema within the interstitium (Kent et al., 2007).

4.3.4 Inotropes

At birth, there is a marked change in hemodynamics, with a subsequent rise in blood pressure and heart rate (Teitel et al., 1987; Louey et al., 2000). Preterm birth causes an abrupt and premature shift in the circulation from the fetal to postnatal configuration at a time when the cardiovascular system is still relatively immature; as a result, it is often necessary for inotropes to be administered to preterm neonates when blood pressure remains abnormally low after birth (Kluckow and Evans, 2001; Osborn et al., 2002). Given the importance of renal blood flow to growth of the kidney it is important that future research examines how the hemodyamic transition at birth affects the development of the immature renal vasculature and/or nephrogenesis and what effect the administration of inotropes have on the developing kidney.

5. Conclusion

Over the past decade, considerable advances have been made in our understanding of the effects of preterm birth on the developing kidney. Encouragingly, it has clearly been demonstrated that nephrogenesis continues after birth in the preterm neonate, however, glomerular abnormalities are commonly observed. Future research should be directed into the causes of these abnormalities, so that strategies can be implemented to maximise the

number of functional nephrons at the beginning of life in the preterm infant in order to ensure long-term renal health. It is important that renal clinicians are made aware of the potential deleterious effects of preterm birth on developing glomerui, so they are aware of the renal vulnerability in subjects that are born preterm.

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REVIEW 3:

STEREOLOGICAL ASSESSMENT OF RENAL DEVELOPMENT IN A BABOON MODEL OF PRETERM BIRTH

CHAPTER ONE (REVIEW 3) DECLARATION

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

Nature of contribution	Extent of contribution (%)
Wrote the majority of the manuscript	85%

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution (%) student co-authors only
Lina Gubhaju	Provided data, assisted in editorial process	10%
M. Jane Black	Assisted in editorial process	5%

Candidate's Signature [redacted] **Date** 30/04/12

Declaration by co-authors
The undersigned hereby certify that:

(13) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(14) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(15) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(16) there are no other authors of the publication according to these criteria;
(17) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(18) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) **Department of Anatomy & Developmental Biology, Monash University**

	Signature	Date
Lina Gubhaju	[redacted]	24/4/2012
M. Jane Black		24/4/12

In-Depth Topic Review

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Stereological Assessment of Renal Development in a Baboon Model of Preterm Birth

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Key Words
Baboon · Kidney · Nephron · Preterm birth

Abstract
At the time when most preterm babies are delivered, nephrogenesis is still ongoing, with the majority of nephrons normally formed during the third trimester of pregnancy. The extrauterine environment, however, is suboptimal for organogenesis, and therefore renal development is likely to be adversely affected by preterm birth. In the long-term, there is emerging evidence of high blood pressure and renal dysfunction amongst young adults born preterm. There is little knowledge to date, however, regarding the effects of preterm birth on renal structural development, perhaps due to the lack of an appropriate animal model. We have demonstrated that the baboon (*Papio* sp.) has a similar time course of nephrogenesis as the human kidney, and the baboon neonate can also be cared for in the same manner as a human neonate following preterm birth. Through a series of studies assessing renal development in the baboon model of preterm birth, involving the use of gold-standard stereological techniques, we have demonstrated that nephron endowment in the preterm baboon kidney is not reduced. Furthermore, antenatal glucocorticoid exposure prior to preterm delivery was associated with an increase in mature nephrons. There was, however, evidence of morphological abnormalities in a variable percentage of the glomeruli formed ex utero. Further research is therefore essential in order to establish what factors are involved in contributing to the glomerular abnormalities, and to identify ways in which 'normal' renal development can be conserved and optimised in the extrauterine setting.

Introduction

Preterm birth (birth prior to 37 completed weeks of gestation) is a significant medical problem worldwide, with up to 13% of babies born preterm in the USA [1], and 8% in Australia [2]; incidence rates have increased 14% in the USA since 1990 [3]. Due to advances in clinical care practices, the survival of preterm neonates is now as high as 80% in babies born as early as 25 weeks of gestation [4, 5]. Birth at a young gestational age is, however, associated with significant morbidity, primarily due to organ immaturity [6]. Importantly, emerging epidemiological research has also indicated that preterm birth is linked to the development of disease in adulthood. Strong associations between low gestational age at birth and high blood pressure [7–17] as well as some evidence of renal dysfunc-

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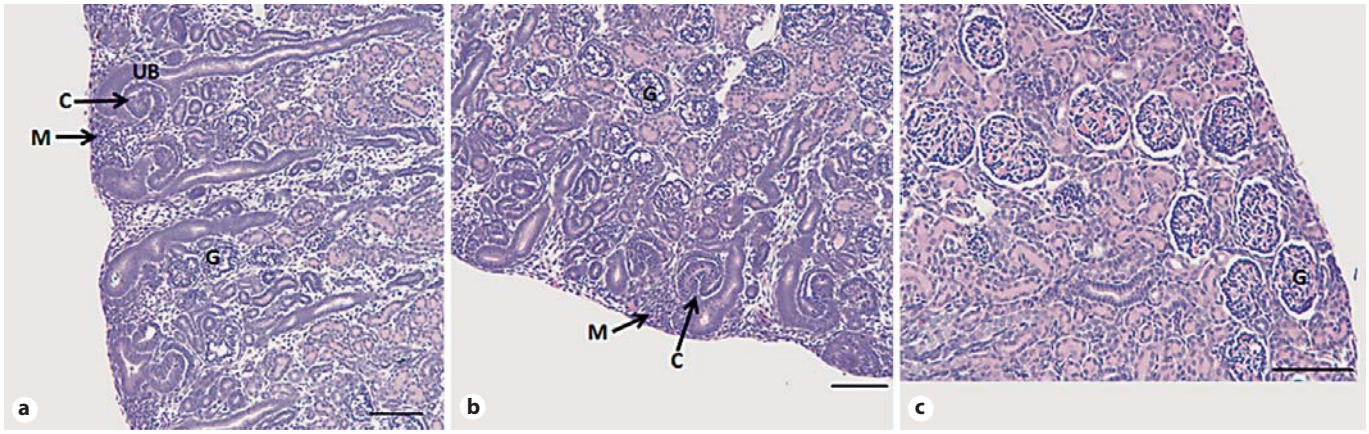


Fig. 1. Photomicrographs demonstrating the time course of nephrogenesis in the fetal baboon kidney. **a** At 125 days of gestation, there is a wide active nephrogenic zone in the outer renal cortex. **b** At 140 days of gestation, nephrogenesis is still ongoing as evidenced by the presence of an active nephrogenic zone. **c** By 175 days of gestation, nephrogenesis is complete with only mature glomeruli located in the superficial layer of the renal cortex. C = Comma-shaped body; G = mature glomerulus; M = metanephric mesenchyme; UB = ureteric bud. Bar = 100 μm.

tion [18–20] suggest the possibility that an impairment in renal development may be underlying these conditions. Nephrogenesis is ongoing until approximately 36 weeks of gestation and is therefore completed before birth in the term infant. The majority of nephrons (approximately 60%) are formed during the third trimester of pregnancy [21]; this coincides with the timing of preterm birth. Therefore, it is conceivable that renal development is adversely affected in infants born prior to the completion of nephrogenesis, with the final stages of nephrogenesis undertaken in the extrauterine environment.

The renal structural consequences of preterm delivery still remain relatively unknown, perhaps due to the lack of an appropriate animal model. Therefore, the purpose of this review is to provide an overview of recent stereological studies of renal development in a baboon model of preterm birth. These findings highlight the importance of continued research in this area, and demonstrate the advantages of utilising the baboon model in renal development research.

The Baboon as a Model of Human Renal Development

Through stereological analyses, we have demonstrated that the baboon (*Papio* sp.) is an ideal animal model for studies of renal development [22]. Similar to the human, the baboon has a relatively long gestation period (approximately 185 days). Furthermore, the ontogeny of organs such as the kidney is very similar to the human [23].

Embryological studies of baboon development determined that nephron formation begins at approximately day 30 of gestation [23], which coincides with the timing of initial ureteric branching in the human kidney [24]. Examination of renal histological sections from baboon fetuses, at varying time points in gestation, further highlighted the parallels in the time course of nephrogenesis between humans and baboons [22]. Nephrogenesis was shown to be ongoing at 125 and 140 days of gestation, with the presence of an active nephrogenic zone (branching ureteric bud and metanephric mesenchyme) in the outer renal cortex, but complete by 175 days, prior to term birth (fig. 1).

In our laboratory, nephron number (a key measure of renal functional capacity) was determined at the later stages of gestation using the physical disector/fractionator approach (described in a later section), which is the gold-standard stereological technique for the unbiased determination of nephron endowment [25, 26]. In fetal baboons at 125 days of gestation, the beginning of the third trimester of pregnancy, nephron endowment averaged at 115,526 per kidney. By 140 days of gestation, nephron number had increased to >160,000 [22]. In baboons where nephrogenesis had been completed (175 and 185 days gestation), total nephron endowment averaged at 284,804 per kidney. There was a strong correlation be-

tween nephron number and kidney weight in the baboon suggesting that renal size is a determinant of nephron number.

Importantly, nephron number at 125 days of gestation averaged at less than half of the total reached by the completion of nephrogenesis [22]. Therefore, similar to the human kidney, the majority of nephrons are formed during the final trimester of pregnancy. Given the similarities in the time course of nephrogenesis between the baboon and the human, the baboon is therefore an ideal model for research into the effects of preterm birth on the kidney.

The Need for an Animal Model of Preterm Birth

There has been just one study published to date (besides case studies) that has reported on the renal structural effects of preterm birth in the human neonate. In this autopsy study by Rodriguez et al. [27], the kidneys of preterm neonates, with varying lengths of postnatal survival, were examined using histomorphometric techniques. Results of these analyses indicated that preterm neonates had a significantly reduced number of medullary ray glomerular generations (an index of nephron endowment [28]) compared to term-born controls; this is potentially indicative of a nephron deficit.

There are difficulties in interpreting these results, however, due to the inherent confounders associated with conducting a human autopsy study. These include intrauterine and extrauterine growth restriction of the neonate, unknown maternal nutritional status and drug use, and the use of an intrinsically ill non-surviving population, all factors which are likely associated with impaired renal development and function in the neonate [29, 30]. Therefore, the use of an appropriate animal model and comprehensive stereological techniques are essential in order to eliminate these confounders and identify the specific effects of preterm birth on renal development.

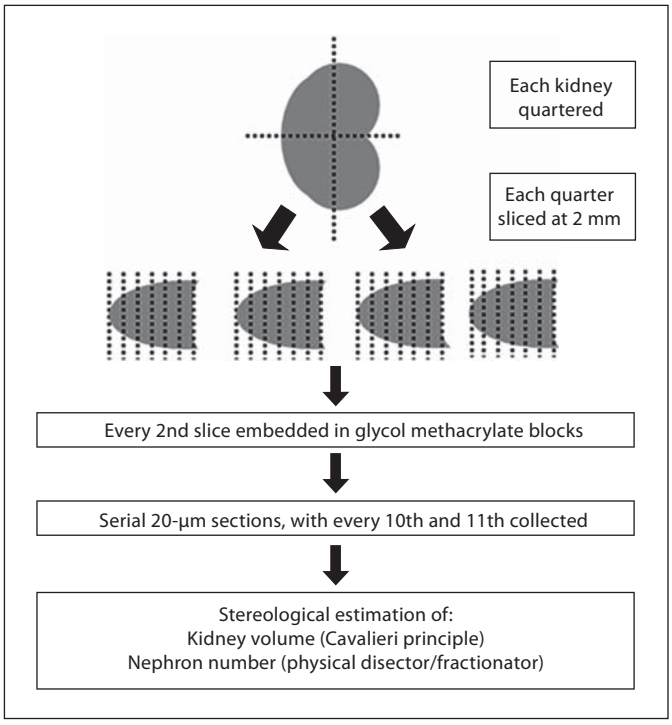
At the Southwest Foundation for Biomedical Research (San Antonio, Tex., USA), a baboon model of preterm birth was developed. Prior to preterm delivery (48 and 24 h), pregnant dams were administered 6 mg of betamethasone. Baboon neonates were then delivered by caesarean section at 125 days (67%) of gestation, which is equivalent to approximately 27 weeks of gestation in humans. Maternal nutrition was consistently maintained, with all neonates born at an appropriate weight for gestational age. Gestational control animals were delivered and euthanized at 125, 146 and 175/185 days of gestation. Further preterm baboon neonates were maintained postnatally and euthanised on day 6, 14 or 21 of life. Importantly, baboon neonates (weighing approximately 1 kg at term) are of a similar size to human preterm neonates, which enables the replication of existing intensive care techniques in their postnatal care.

The practice of rapid weaning from ventilatory support, early introduction of parenteral nutrition, and minimal handling were used in the care of the preterm baboon neonates [31]. Briefly, neonates were intubated following delivery, administered surfactant and ventilated with a humidified pressure-limited and time-cycled ventilator. Extubation to nasal continuous positive airway pressure was attempted at 24 h of age. Parenteral nutrition was initiated at 24 h of life and if clinically stable, enteral nutrition was introduced on day 7. Serum electrolytes, glucose, and haematocrit were maintained within the normal range for the extremely-low-birth-weight infant. None of the animals had any identifiable urinary tract anomalies or obstructions, and all were in relatively good health at the time of necropsy. For a more detailed report on the neonatal care of the baboons, see Thomson et al. [31].

Stereological Assessment of Renal Development

Given that nephrogenesis is ongoing at the time of preterm delivery in the baboon neonate, with the majority of nephrons normally formed during the final trimester of pregnancy [22], we hypothesised that nephrogenesis would be adversely affected by preterm birth. Therefore, we utilised various stereologic and histomorphometric techniques to assess glomerular generation number, the percentage of abnormal glomeruli, nephron number, kidney volume and renal corpuscle volume [22, 32, 33].

At necropsy, the baboon kidneys were excised, cut into quarters along the coronal and horizontal planes, and immersion fixed. The kidney quarters were then sampled using the fractionator principle [34]. Each quarter was sliced into 2-mm slices, and every 2nd slice (sampling fraction F_1) was embedded in glycol methacrylate resin (1 slice per resin block). The glycol methacrylate blocks were serially sectioned at 20 μm; every 10th pair of sections (sampling fraction F_2), commencing at a random starting point between 1 and 10 (for example, sections numbered 6, 7, 16, 17, 26 and 27), was collected and stained with haematoxylin and eosin. Total kidney volume (V_{kid}) was then estimated using the Cavalieri principle [35]. To do this, every 10th section was viewed using a microfiche reader, with the projected image overlaid by a 2 × 2 cm unbiased



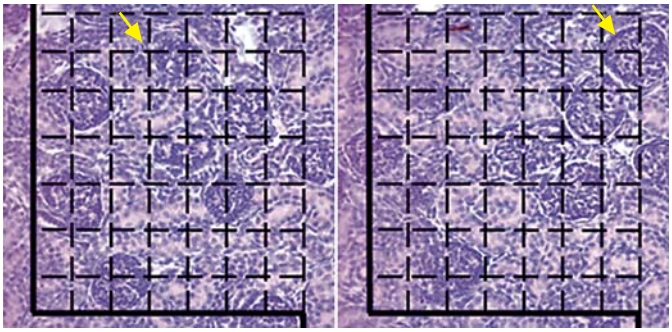


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Fig. 2. The physical disector/fractionator technique was used for the estimation of nephron endowment. **a** The stages of tissue sampling using the fractionator principle for the estimation of kidney volume (using the Cavalieri principle) and nephron number (using the physical disector/fractionator technique). **b** Representative adjacent 10th and 11th baboon kidney sections overlaid with unbiased 64-point counting frames. The two sections were compared and mature glomeruli were counted if they were present in the section on the right and not on the left, and vice versa, within the unbiased counting frames (partly or wholly within the dashed lines and not touching the bold lines). The glomeruli counted (indicated by arrows; Q^-) were used in the estimation of nephron number using the physical disector/fractionator approach.

orthogonal grid. The number of intersecting grid points overlaying the kidney tissue on each section was recorded, and total kidney volume was then estimated using the following equation:

$$V_{kid} = \frac{1}{F_1} \times \frac{1}{F_2} \times t \times a(p)_M \times P_s \quad (1)$$

where $1/F_1$ and $1/F_2$ are the inverse sampling fractions at each level of tissue sampling; t is the thickness of the sections; $a(p)_M$ is the area associated with each intersecting grid point, and P_s is the total number of intersecting points counted per kidney. The number of intersecting grid points overlaying one intact section per slice to be used in the estimation of nephron number was also recorded as P_f.

Total nephron number was estimated using the physical disector technique. This is an unbiased approach whereby each glomerulus (regardless of size, shape and position) has an equal chance of being sampled [25, 26]. Two modified microscopes with projection arms were used to compare images of paired 10th and 11th histological sections (one completely intact pair of sections per slice of kidney sampled; approximately 8 per kidney). Paired images were projected onto two identical and unbiased 64-point counting frames (fig. 2). Paired kidney sections were systematically sampled (commencing at a random starting point at the top of the section) in a uniform pattern along the x- and y-axis. At each field of view, the number of intersecting grid points overlaying the kidney tissue (P_{kid}) and renal corpuscles (P_{corp}) were recorded. The number of glomeruli present in the projected image of the 10th section, but not in the matched 11th section (within the parameters of the unbiased counting frame) and vice versa is recorded as a total of Q^- at each field of view. Importantly, only mature glomeruli were counted, with developing structures such as comma- and S-shaped bodies being excluded. The total number of glomeruli in the kidney (N_{glom}) was estimated using the following calculation:

$$N_{glom} = \frac{1}{F_1} \times \frac{1}{F_2} \times \frac{P_s}{P_f} \times \frac{1}{2f_a} \times Q^- \quad (2)$$

where $1/F_1$ and $1/F_2$ refer to the inverse sampling fractions at each level of tissue sampling; P_s/P_f is the inverse of the sampling fraction relating to the kidney sections analysed; Q^- is the total number of glomeruli counted per kidney, and $1/2f_a$ is the inverse of the fraction of the total tissue area used to count glomeruli, where f_a is calculated in the following manner:

$$f_a = \frac{P_{kid} \times a(p)_{PD}}{P_f \times a(p)_M} \quad (3)$$

P_{kid} is the total number of intersecting grid points counted per kidney; $a(p)_{PD}$ is the area associated with each intersecting grid point used with the physical disector; P_f is the number of intersecting grid points on the one intact section per slice of kidney used with the physical disector, and $a(p)_M$ is the area associated with each grid point used on the microfiche.

The mean renal corpuscle volume (V_{corp}) was calculated by dividing the volume density of the renal corpuscle by the numerical density of renal corpuscles within the kidney:

$$V_{corp} = \frac{P_{corp}}{P_{kid}} \div \frac{N_{glom}}{V_{kid}} \quad (4)$$

During the assessment of nephron number, the number of morphologically abnormal and normal glomeruli was also recorded at each field of view. The total percentage of abnormal glomeruli (exhibiting an enlarged Bowman's space and shrunken glomerular tuft) was then calculated per kidney.

This comprehensive assessment of renal development was possible due to whole kidneys (or a known fraction of the kidney) being available for analysis; this is often not the case following human autopsy. The medullary ray glomerular generation counting method is a useful technique for the assessment of nephrogenesis in the absence of whole kidneys [28]. This method involves identifying clearly distinguishable medullary rays in histological renal sections, and counting all developed glomeruli along one side of the ray, in a line from the inner to outer renal cortex. In our baboon studies, we have found a strong correlation between glomerular generation number and nephron number, which supports the validity of the technique (fig. 3).

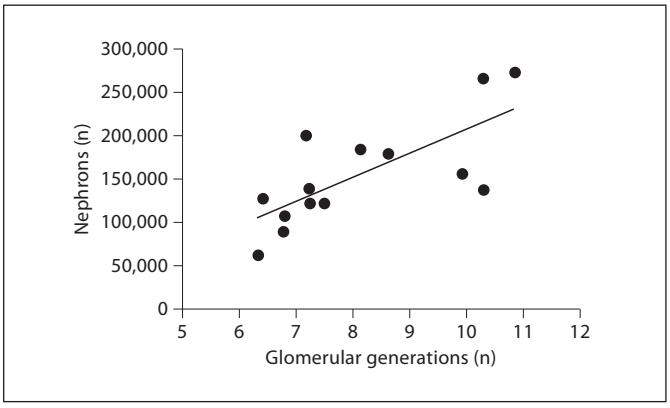

Fig. 3. There is a significant linear correlation between glomerular generation number and nephron number ($R^2 = 0.536$, $p = 0.003$) in the developing baboon kidney (derived from Gubhaju et al. [22]).

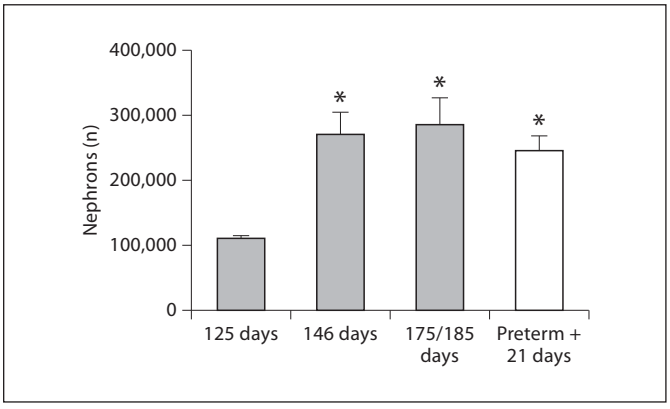

Fig. 4. Average nephron numbers in fetal baboons at 125, 146 and 175/185 days of gestation, and in preterm baboons on postnatal day 21. Nephron number had significantly increased since the time of delivery in the preterm group; however, it was not significantly different to the age-matched gestational controls (146 days) and term (175/185 days) controls (derived from Gubhaju et al. [32]). * $p < 0.05$ vs. the 125-day group.

The Effects of Preterm Birth on Renal Development

Our recently published data from stereological analyses of the preterm baboon kidney [32] indicated that, in comparison to age-matched gestational controls, there was no significant difference in nephron number or glomerular generation number in the preterm kidney (fig. 4); this is in contrast to the findings from the previously published human autopsy study by Rodriguez et al. [27]. Nephron number in the preterm baboon kidney appeared to be within the normal range, albeit at the lower end; however, nephrogenesis was still ongoing at the time of analysis, so nephron number was expected to increase further before the completion of nephrogenesis. This difference in results compared to the previously published autopsy study [27] is likely due to their inclusion of intrauterine-growth-restricted neonates, a factor known to significantly impair nephrogenesis [36, 37].

There was substantial kidney growth in the postnatal period following preterm delivery, with significantly larger kidneys in relation to body weight in the preterm baboon compared to age-matched gestational controls [32]. Importantly, the strong correlation between nephron number and kidney weight/volume (noted in previous studies in the baboon [22]) was maintained in the

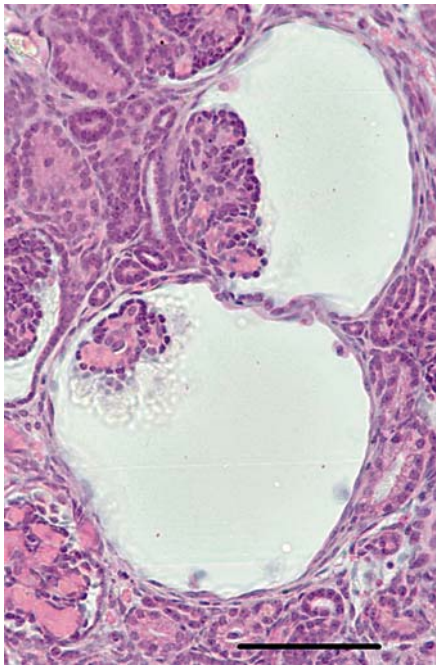

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Fig. 5. Representative photomicrograph of abnormal glomeruli (with shrunken glomerular tufts and dilated Bowman's spaces) in the outer renal cortex of a preterm baboon kidney. Bar = 100 μm.

preterm kidney [32]. However, glomerular density (number of glomeruli per gram of kidney tissue) was significantly reduced in the preterm kidney (83,840 glomeruli/g) compared to controls (193,400 glomeruli/g). Given that total nephron number was not reduced and renal corpuscle size not affected [32], the reduction in glomerular density most likely reflects a postnatal increase in tubular or interstitial mass.

Of major concern, a significant proportion of morphologically abnormal glomeruli, which exhibited an enlarged Bowman's space and shrunken glomerular tuft, were commonly noted in the outer renal cortex of the preterm baboon kidney (fig. 5) [32]. Similar findings have been reported in the human preterm kidney [27]. Importantly, given the localisation of the affected glomeruli in the outer renal cortex, it is likely that it is those glomeruli that are newly formed in the extrauterine environment that are vulnerable.

There was a large variability between neonates in the proportion of abnormal glomeruli present, ranging from 0.2 to 18.3% [32]. This suggests that the abnormalities may not have occurred as a result of preterm birth per se, but are likely related to factors in their postnatal care, which varied between individual neonates. For example, neonatal exposure to nephrotoxic medications (such as non-steroidal anti-inflammatory drugs and antibiotics) has been associated with both structural and functional renal injury [38]. However, a specific factor attributing to the abnormal glomeruli has not yet been established. Given the extent of the abnormality, it is likely that the affected glomeruli will never become functional, perhaps resulting in a deficit of functional nephrons, as noted in the human preterm kidney by Rodriguez et al. [27].

Antenatal Glucocorticoid Administration

One factor which is commonly used in the care of the preterm neonate is the administration of antenatal glucocorticoids. In clinical practice, the glucocorticoids betamethasone or dexamethasone are administered to pregnant women at risk of preterm delivery in order to accelerate fetal lung maturation [39]. Importantly, exposure to glucocorticoids antenatally is also associated with increased mean arterial blood pressure, renal blood flow and glomerular filtration rate [40–46], suggesting that accelerated renal functional maturation also occurs.

Through previous stereological analyses using animal models such as the rodent [47–49] and sheep [50], studies have indicated that a reduced nephron endowment in offspring is associated with antenatal glucocorticoid exposure. Furthermore, a recent study by de Vries et al. [51] found postnatal administration of dexamethasone in the neonatal rat (at a time of ongoing nephrogenesis) led to a reduction in glomerular density. Our studies in the preterm baboon model involved replication of the clinical setting, with 0.4 ml/kg/day of betamethasone administered to pregnant baboons 48 and 24 h prior to delivery [32]. Assessment of the kidneys from these offspring, at 125 days of gestation (the time of caesarean section delivery) and 21 days postnatally, demonstrated that nephron endowment was not adversely affected by steroid exposure. Importantly, structural evidence of enhanced renal maturation was evident, with an increase in the number of mature nephrons present in the steroid-exposed kidneys compared to those that were not exposed (fig. 6) [32]; this parallels the findings of increased renal functional capacity following antenatal glucocorticoid exposure. These results imply that renal development is not likely to be adversely affected in the short term by this common clinical procedure, which is vital for postnatal survival of the preterm infant. As nephrogenesis was still ongoing at the time of assessment, however, it is unknown whether there are long-term effects

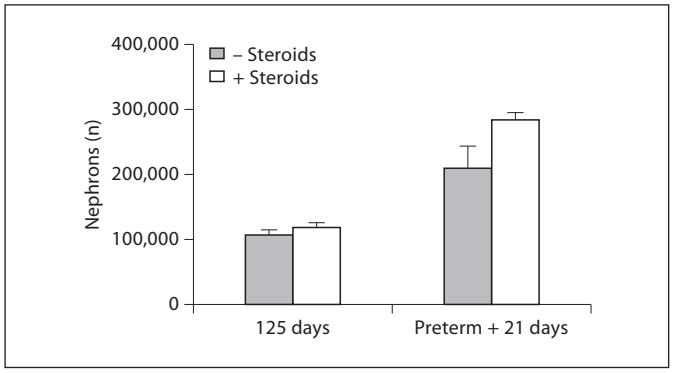

Fig. 6. Average nephron number in fetal baboons at 125 days of gestation and in preterm baboons on postnatal day 21 that were exposed or not exposed to antenatal steroids. Two-way ANOVA analysis determined that antenatal steroid exposure ($p = 0.03$) and increased postnatal age ($p < 0.0001$) were both associated with a significant increase in nephron endowment (derived from Gubhaju et al. [32]).

of the accelerated renal maturation, such as the early cessation of nephrogenesis, which may influence total nephron endowment.

Postnatal Retinoic Acid Administration

It can be hypothesised that a preterm kidney with a large proportion of abnormal glomeruli may ultimately suffer a nephron deficit, hence it is important to maximise nephron endowment in the preterm infant. Theoretically, there is a critical 'window of opportunity' in the early postnatal period following preterm birth whereby interventional strategies may be implemented in order to enhance nephrogenesis. One potential strategy that has been investigated was the administration of retinoic acid, the active metabolite of vitamin A. Importantly, vitamin A administration is already being trialled in preterm infants due to successful outcomes in reducing neonatal morbidity and mortality [52]. In the kidney, the administration of retinoic acid upregulates the expression of the c-ret tyrosine kinase receptor, resulting in an increase in ureteric bud branching and thereby increasing the number of nephrons formed [53, 54]. Importantly, the antenatal administration of retinoic acid in a rodent model resulted in augmented nephron endowment in the offspring, even to supernumerary levels [55], and furthermore subjugated the nephron deficit expected in offspring with simultaneously imposed intrauterine growth restriction [56].

In the baboon model of preterm birth, 500 μg/kg of retinoic acid was administered daily to neonates following preterm delivery at 125 days of gestation. Through stereological analyses of renal development in the 21-day-old preterm baboons, it was determined that postnatal retinoic acid administration did not influence total nephron number or the number of glomerular generations. This lack of effect of retinoic acid on nephron endowment is likely due to the late timing of administration. In the human kidney, ureteric bud branching is completed by approximately 22 weeks of gestation [57] (near the borderline of viability in the preterm infant), therefore only exposure to retinoic acid prior to this time would be likely to enhance nephrogenesis. Therefore, any postnatal interventional strategies in the preterm infant must be focused on the later stages of renal development, such as glomerular generation formation, rather than on the period of branching morphogenesis.

Conclusion

We have shown through a series of stereological studies, conducted using a clinically relevant baboon model, that nephrogenesis continues following preterm birth; however, it may be impaired with evidence of morphological glomerular abnormalities in the outer cortex of some, but not all, preterm kidneys. Importantly, antenatal glucocorticoid exposure prior to preterm birth is associated with accelerated renal maturity, which may impart a benefit on renal function in the early postnatal period. It is essential that future research be focused on determining which factors in the postnatal care of the preterm infant result in impaired renal development, and on ways in which to provide the optimal environment in the neonatal intensive care unit for postnatal organogenesis to occur.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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CHAPTER TWO

ASSESSMENT OF GLOMERULAR AND TUBULAR FUNCTION, AND A MARKER OF RENAL INJURY, IN PRETERM NEONATES DURING THE FIRST MONTH OF LIFE

CHAPTER TWO DECLARATION

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

Nature of contribution	Extent of contribution (%)
Performed experimental work for ~60% of babies, all data analysis, and co-authored manuscript	50%

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution (%) For student co-authors only
Lina Gubhaju	Set up studies, performed experimental work for ~40% of babies, co-authored manuscript	40%
Rosemary Horne, Alison Medhurst, Alison Kent, Andrew Ramsden, Lynette Moore, Wendy Hoy, M. Jane Black	Involved in the design of experiments, and assisted in editing the manuscript	10%

Candidate's Signature [redacted] **Date** 30/04/12

Declaration by co-authors

The undersigned hereby certify that:

(19) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(20) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(21) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(22) there are no other authors of the publication according to these criteria;
(23) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(24) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) **Department of Anatomy & Developmental Biology, Monash University**

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ASSESSMENT OF GLOMERULAR AND TUBULAR FUNCTION, AND A MARKER OF RENAL INJURY, IN PRETERM NEONATES DURING THE FIRST MONTH OF LIFE

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ABSTRACT

Worldwide, approximately ten percent of neonates are born preterm. Preterm neonates are born when the kidneys are still developing, and therefore, renal function in the early postnatal period is likely affected by both renal immaturity and injury. The aim of this study was to evaluate glomerular and tubular function, and a marker of renal injury (NGAL), in preterm neonates during the first month of life. Preterm neonates were grouped according to gestational age at birth: ≤ 28 weeks (extremely preterm; n=33), 29 – 31 weeks (very preterm; n=44), 32 – 36 weeks (moderately preterm; n=32), and ≥ 37 weeks (term; n=22). Measures of glomerular function (creatinine clearance, urine albumin, and urine total protein) and tubular function (the fractional excretion of sodium and urine β2-microglobulin) were assessed at postnatal days 3-7, 14, 21 and 28 of life. Overall, the findings of this study demonstrate that glomerular and tubular function are significantly affected by gestational age at birth, as well as postnatal age, suggesting that neonatal renal function is predominantly influenced by renal maturity. Urinary NGAL did not appear to be an accurate predictor of acute kidney injury in the preterm neonate; however, severe proteinuria and high urinary NGAL levels were observed in a number of preterm neonates which may be indicative of renal injury in the early postnatal period.

INTRODUCTION

Renal function in the preterm neonate is affected by renal immaturity and potential injury during the early postnatal period. At the time of preterm birth, renal development is still ongoing (58) and renal function is accordingly immature (20). Preterm neonates have been shown to have a low glomerular filtration rate (GFR) compared to term neonates, and the tubules excrete high amounts of sodium (3). Furthermore, compared to babies born at term, preterm neonates may demonstrate a slower progression in renal functional maturation after birth (7, 19). Creatinine clearance (C_{Cr}; an indicator of GFR) shows a positive correlation with both gestational age and postnatal age (3, 7, 11, 12, 14, 17, 19, 29, 48, 57, 61, 67), while the fractional excretion of sodium (FE_{Na}; an indicator of tubular function) shows an inverse correlation with gestational age (17) and postnatal age (3, 17, 18, 48, 57).

Only a few studies to date have investigated the occurrence of proteinuria following preterm birth. Very low concentrations of both high molecular weight (HMW) and low molecular weight (LMW) proteins are normally present in the urine, due to the function of the glomerular filtration barrier and the reuptake of filtered proteins in the proximal tubule (37, 41). Previous studies have demonstrated that there is a high variation in urine albumin (HMW protein) levels between individual neonates (11, 15), with the highest levels exhibited by those with a low gestational age at birth and those that are not clinically stable (6, 11, 15, 16, 62). Urinary levels of LMW proteins (such as β2-microglobulin) have also been shown to be significantly greater in the preterm infant compared to term-born infants throughout the first month of life (3, 62, 63), and they are decreased with increasing gestational and postnatal age (60). To date, however, it remains unclear whether the increased urinary high- and low- molecular weight protein levels reported in preterm babies are associated with renal immaturity and/or occur due to postnatal renal injury.

The kidney of the preterm neonate is very susceptible to injury in the neonatal period. Acute kidney injury (AKI) is reported to occur in 8% to 24% of preterm neonates admitted to neonatal intensive care units (27, 54), and is primarily pre-renal in origin (8, 54). In a large study of preterm infants born in the USA and Puerto Rico, Walker *et al*. (66)

examined the medical records of 66,526 neonates (born at ≤ 30 weeks gestation); 4% of the neonates were diagnosed with renal dysfunction and/or renal failure. The predominant risk factors for impaired renal function were low gestational age and low birth weight. Further risk factors included postnatal medication administration (vasopressor, indomethacin, and antibiotics), postnatal illness (intraventricular haemorrhage, a patent ductus arteriosus, necrotising enterocolitis, culture positive sepsis) and also the use of high frequency ventilation, male gender and non-white race. Importantly, mortality rates were significantly higher in neonates with the diagnosis of renal dysfunction and/or renal failure (66). In addition, renal injury in the preterm neonate may be an antecedent to chronic renal disease (2). Therefore, the early diagnosis and treatment of AKI is essential to the preterm neonate for the maintenance of both short and long-term renal function. In this regard, urinary neutrophil gelatinase associated lipocalin (NGAL) has recently been investigated as a potential biomarker of AKI in preterm neonates, with studies showing that NGAL levels strongly correlated with both gestational and postnatal age (28, 32), and are highest in neonates that are critically ill (32, 44).

In this Australian-based study, we have examined renal function in preterm infants admitted to the neonatal intensive care unit at the Monash Medical Centre (a large tertiary level hospital, located in Melbourne, Australia); the current rate of preterm birth in the Australian population is 8.2% (33). The aims of the study were to: 1) assess postnatal renal function from day 3 to day 28 in moderately preterm (32 – 36 weeks of gestation), very preterm (29 – 31 weeks of gestation) and extremely preterm (≤ 28 weeks of gestation) neonates by examining glomerular (C_{Cr}, and urine albumin) and tubular (FE_{Na} and urine β2-microglobulin) function; and 2) determine whether urinary NGAL is a useful marker of renal injury in preterm neonates.

METHODS

Ethics approval for this study was obtained from the Southern Health Human Research Ethics Committee and the Monash University Standing Committee on Ethics in Research Involving Humans. Written informed parental consent was obtained for all participants in the study.

Recruitment of participants

Neonates were recruited from Monash Medical Centre and Jessie MacPherson Private Hospital (Clayton, Victoria, Australia). All neonates were born between April 2008 and October 2011. Preterm neonates born at less than 37 weeks gestation, and term neonates born between 37 and 42 weeks gestation, without any congenital abnormalities, were eligible for the study. The neonates were stratified into the following gestational age groups: Group A (extremely preterm; ≤ 28 weeks), Group B (very preterm; 29 – 31 weeks), Group C (moderately preterm; 32 – 36 weeks), and Group D (term; ≥ 37 weeks). Complete maternal and neonatal medical records were recorded for each neonate.

Urine collection procedure

Urine samples were obtained from sanitary pads (Kotex; Kimberly-Clark, NSW, Australia) placed within the nappy of the neonates, a method that has been previously validated (26). A nappy liner (Johnson's Baby Nappy Liners; Johnson & Johnson Pacific, NSW, Australia) was also placed inside the nappy to filter out any faeces. All nappies inclusive of the pad and nappy liner were weighed before they were put on the neonate and weighed again after use in order to determine urine output (47). Urine was extracted by compressing the sanitary pad using a hydraulic press (26).

It has been shown that urine collected from disposable pads and/or cotton wool does not affect the urinary constituent of sodium, potassium or creatinine (47) and has been used previously in the analysis of urinary NGAL (32). However, previous research has shown that protein gets trapped in cotton material (53). Therefore, clean spot urine samples, collected using urine collection bags, were utilised to determine urinary total protein and urinary albumin and β2-microglobulin levels.

In preterm neonates, urine collection began at 72 hours following the time of birth (day 3) and continued until postnatal day 7. In addition, urine was collected for a 24 hour period on days 14, 21 and 28 of life. Spot urine samples (approximately 2 ml) were obtained on days 7, 14, 21 and 28 of life. In term neonates, urine collection commenced 48 hours after birth (day 2) and continued until the infant was discharged from hospital (approximately day 4 of life). Additionally, urine was collected for a 24 hour period on day 28 of life. Spot urine samples were obtained from the term neonates on day 3 of life. In instances where infants were discharged from hospital prior to postnatal day 28, nappies were prepared and delivered to parents, and collected the following day. Urine collected over each 24 hour period was pooled for each neonate. The pooled urine was frozen at -20°C until analysis. Spot urine samples were sent for analysis of urinary protein levels immediately after collection.

Assessment of renal function

Urinary and plasma sodium and creatinine

All urine analyses were performed by the Southern Health Pathology Department (Southern Cross Pathology; Clayton, Victoria, Australia). Pooled 24 hour urine samples were analysed for sodium and creatinine levels. Plasma creatinine and plasma sodium levels were recorded from blood tests that were undertaken as part of the routine care of preterm neonates, and data were extracted from the medical records. In term neonates, heel-prick blood samples were obtained for analysis at the time of the routine newborn screening test (at approximately 48 hours of life). In cases where a blood test was not available in term infants on postnatal day 28, average levels of plasma sodium (140 mmol/L) and plasma creatinine (40 μmol/L) that are within the expected range for term infants were used in the calculation of C_{Cr} and FE_{Na}.

Calculation of C_{Cr} and FE_{Na}

Creatinine Clearance (C_{Cr}) was calculated using the following formula:

C_{Cr} *(ml/min/BSA) = (UCr * Urine Flow Rate * PCr) / BSA*

Where UCr = urinary creatinine (µmol/L); Urine Flow Rate = ml/min (calculated for each 24 hr period); PCr = plasma creatinine (µmol/L); BSA = body surface area (m^2).

Body surface area (BSA) was calculated using the following formula derived by Haycock *et al*. (24):

$$BSA\ (m^2) = 0.007184 * body\ weight\ (kg)^{0.425} * body\ length\ (cm)^{0.725}$$

The fractional excretion of sodium (FE_{Na}) was calculated using the following formula:

$$FE_{Na}\ (\%) = (UNa / PNa) * (PCr / UCr) * 100$$

Where UNa = urinary sodium (mmol/L); PNa = plasma sodium (mmol/L); PCr = plasma creatinine (µmol/L); UCr = urinary creatinine (µmol/L).

Urinary total protein, albumin and β2-microglobulin

Spot urine samples were used to measure levels of urine total protein (UTP), urinary albumin and urinary β2-microglobulin. Urine protein levels were measured using nephelometric technology on a Beckman immunochemistry system, with reagents and calibrators supplied by Beckman Diagnostics (urine total protein and urine albumin; Beckman Diagnostics; Sydney, Australia) and DakoCytomation (β2-microglobulin; DakoCytomation; Glostrup, Denmark). In instances where urine total protein was greater than 500 mg/L, this was defined as pathological proteinuria and urinary albumin levels were not determined. All urine protein levels were expressed as a ratio to urine creatinine concentrations.

Urinary NGAL

Pooled 24 hour urine samples were analysed for urinary NGAL levels in 79 (61.2%) neonates at one or more postnatal time points. NGAL analysis was performed using a sandwich ELISA in microwells coated with a monoclonal antibody against human NGAL (NGAL ELISA Kit, BioPorto Diagnostics A/S; Gentofte, Denmark); the upper limit of the test was 500 ng/ml.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v5.04 for Windows (CA, U.S.A.) and Intercooled Stata v8.0 for Windows (TX, USA). Data are presented as the mean ± standard error of the mean (SEM). Statistical significance was accepted at the level of $p<0.05$.

Birth characteristics (gestational age, birth weight, length, head circumference) were compared among groups using a one-way analysis of variance (ANOVA), followed by a Bonferroni post-hoc test. To determine differences in categorical variables (such as sex, disease outcomes, and medication administration) among groups, a Fisher's exact test was performed.

Urine output, creatinine clearance and the fractional excretion of sodium in preterm neonates (Groups A, B and C) were analysed using a two-way ANOVA with repeated measures. At postnatal day 3 and day 28, urine output, creatinine clearance and the fractional excretion of sodium (in all four groups) was analysed using a two-way ANOVA, followed by a Bonferroni post-hoc test to determine differences between individual groups at each time point. Urine protein (urine total protein, albumin, and β2-microglobulin) to creatinine ratios and urine NGAL levels were also analysed using a two-way ANOVA, followed by a Bonferroni post-hoc test. The factors assessed in all of these analyses were gestational age (p_{GA}), postnatal age (p_{PA}) and their interaction ($p_{GA \times PA}$).

Linear regression analyses (followed by an analysis of covariance (ANCOVA)) were used to compare the rate of change in C_{Cr} and FE_{Na} from postnatal day 3 to day 28, and also to determine whether urinary NGAL levels correlated with any other indices of renal function (Urinary NGAL levels > 390 ng/ml were excluded as outliers). Additionally, urinary NGAL levels in neonates exhibiting pathological proteinuria (UTP ≥ 500 mg/L) were compared to age and sex-matched neonates (controls) with low urine total protein levels (UTP < 500 mg/L, and at least half the level of the age-matched neonate with proteinuria) at the corresponding time point. This analysis was undertaken using a two-way ANOVA with the factors proteinuria (p_P), gestational age (p_{GA}) and their interaction ($p_{P \times GA}$). Additionally, in these two groups, urinary NGAL levels were assessed at time points prior to (7 days prior if pathological proteinuria observed at day 14, 21 or 28; 3

days prior if observed at day 7), and at the time of proteinuria onset. This analysis was undertaken using a two-way ANOVA with the factors proteinuria (p_P), time point of assessment (p_T) and their interaction ($p_{P \times T}$).

Impaired renal function (low urine output, low creatinine clearance, high fractional excretion of sodium, high urine total protein and high urinary NGAL) was defined as values greater than 2 standard deviations from the mean at any time point from postnatal day 3 through to day 28; the mean and standard deviation was calculated from values inclusive of all preterm neonates (Groups A, B and C).

RESULTS

Study population

Neonates were recruited between April 2008 and October 2011. During this time, 196 parents were approached to participate in the study. Fifty-three parents refused to have their baby participate; the majority of refusals to participate were from the parents of term infants (Group D). In all, 143 neonates were recruited to participate. After the commencement of the study, four neonates were excluded following the early withdrawal of parental consent. Eight preterm neonates were further excluded following transfer to other hospitals prior to postnatal day 7. Two preterm neonates (born ≤ 25 weeks gestation) died before the study was completed.

The resultant study population consisted of 129 neonates that were stratified into four groups according to gestational age: Group A (≤ 28 weeks gestation; n=33), Group B (29 – 31 weeks gestation; n=44), Group C (32 – 36 weeks gestation; n=30), and group D (≥ 37 weeks gestation; n=22). The number of neonates studied after day 14 was lower, primarily due to transfer of infants to private hospitals, or discharge from hospital. Therefore, the sample size at the postnatal day 14-28 time points were reduced: Group A (≤ 28 weeks gestation; n=32/33 (97.0%)), Group B (29 – 31 weeks gestation; n=32/44 (72.7%)), Group C (32 – 36 weeks gestation; n=19/30 (63.3%)), and Group D (≥ 37 weeks gestation; n=11/22 (50.0%)).

There were difficulties in obtaining clean spot urine samples from a number of the infants due to urine bag leakage and parental refusal. The spot samples were only collected at the discretion of nursing staff; in extremely preterm neonates (Group A, ≤ 28 weeks gestation) whose skin was still very fragile, use of adhesive urine bags was inappropriate. Overall, 46.2% of the total number of requested urine samples were obtained. Urine samples at one or more of the postnatal time points were obtained for the majority of neonates: Group A (≤ 28 weeks gestation; n=30/33 (90.9%)), Group B (29 – 31 weeks gestation; n=41/44 (93.2%)), Group C (32 – 36 weeks gestation; n=26/30 (86.7%)), and Group D (≥ 37 weeks gestation; n=6/22 (27.3%)).

Maternal characteristics

The preterm delivery of neonates in Groups A, B and C followed the onset of spontaneous preterm labour (34.0%), premature pre-labour rupture of membranes (PPROM; 30.1%), placenta praevia/abruption (15.5%) or was clinically indicated due to maternal and/or fetal health risks such as preeclampsia and suspected fetal compromise (20.4%). There were a significantly higher percentage of births attributed to spontaneous preterm labour, and a correspondingly lower percentage due to PPROM, in Group A neonates compared to both Group B and Group C neonates. The majority of preterm neonates (> 65%) in groups A, B and C were born via caesarean delivery, whereas 27% of term neonates in Group D were delivered via caesarean (Table 1). At least 70% of the mothers of preterm neonates (Groups A, B and C) received antenatal steroids prior to delivery (Table 1); 94% of those born extremely preterm received antenatal steroids.

Birth characteristics

Birth characteristics of the preterm and term neonates are described in Table 1. Body weight, length and head circumference at birth all significantly increased along with increasing gestational age. There was no significant difference in the gender balance between gestational age groups. Groups A and C had the greatest number of small-for-gestational age (SGA) neonates; there was a similar number of multiple births in each of the preterm groups whereas all term-born neonates in Group D were singletons. The majority of neonates that had a low Apgar score at 5 minutes (≤ 7) were in group A (≤ 28 weeks gestation).

Neonatal complications

As shown in Table 1, the majority of all preterm neonates (groups A, B and C) required mechanical ventilation after birth; respiratory distress syndrome was very common in these groups. The occurrence of culture-positive sepsis and patent ductus arteriosus was significantly greater in Group A compared to those infants in older gestational age groups. Intraventricular haemorrhage only occurred in neonates within Group A and Group B.

Neonatal medications

Table 2 shows the medications administered to neonates in the early postnatal period, and the percentage of neonates per gestational age grouping that were exposed to each type of medication. Term-born infants (Group D) did not receive any drugs over the course of the study. The most commonly administered medications in preterm neonates was a routine regimen of antibiotics which included both benzylpenicillin (60 mg/kg/dose) and gentamicin (4 mg/kg/dose); all neonates in groups A and B received this treatment. Additional antibiotics for the treatment of sepsis and localised infections included vancomycin, erythromycin, cephalosporins and metronidazole. The majority of neonates that received additional antibiotics were in the extremely preterm group (Group A). Other commonly administered medications included antifungal drugs, methylxanthines, inotropes, steroids, diuretics and non-steroidal anti-inflammatory drugs (NSAIDs). In general, the administration of these drugs was significantly greater in group A neonates (Table 2).

Table 1: Characteristics of the study population, medical interventions, and disease outcomes from birth to postnatal day 28.

	Group A ≤ 28 weeks (n=33)	**Group B 29 – 31 weeks (n=44)**	**Group C 32 – 36 weeks (n=30)**	**Group D ≥ 37 weeks (n=22)**
Birth Characteristics				
Gestational Age (weeks) mean ± SEM (range)	26.6 ± 0.2* (24-28)	30.4 ± 0.1* (29-31)	33.7 ± 0.2* (32-36)	39.6 ± 0.2* (37-42)
Birth Weight (g) mean ± SEM (range)	811.1 ± 28.3* (529-1229)	1438.0 ± 39.6* (892-2157)	1771.0 ± 75.4* (1018-2542)	3356.0 ± 76.2* (2820-4160)
Body Length (cm) mean ± SEM (range)	33.6 ± 0.4* (29-39)	40.9 ± 0.4* (36-46)	43.4 ± 0.7* (36-50)	49.9 ± 0.4* (46-55)
Head Circumference (cm) mean ± SEM (range)	23.9 ± 0.2* (21-27)	28.3 ± 0.2* (25-32)	29.9 ± 0.4* (26-33)	34.0 ± 0.3* (32-36)
Male *n* (%)	12 (36.4%)	23 (52.3%)	14 (46.7%)	11 (50.0%)
SGA *n* (%)	10 (30.3%)^BD	3 (6.8%)^A	14 (46.7%)^BD	1 (4.5%)^AC
Twin/Triplet *n* (%)	9 (27.3%)^D	10 (22.7%)^D	12 (40.0%)^D	0 (0.0%)*
Antenatal Steroids *n* (%)	30 (93.8%)^D	34 (77.3%)^D	21 (70.0%)^D	0 (0%)*
Caesarean Delivery *n* (%)	26 (78.8%)^D	29 (65.9%)^D	21 (70.0%)^D	6 (27.3%)*
Postnatal Characteristics and Disease Outcomes				
Apgar ≤ 7 at 5min *n* (%)	15 (45.5%)*	10 (22.7%)*	1 (3.3%)^AB	0 (0.0%)^AB
Mechanical Ventilation *n* (%)	33 (100%)^CD	41 (93.2%)^CD	17 (56.7%)*	0 (0.0%)*
Respiratory Distress Syndrome *n* (%)	26 (78.8%)^CD	28 (63.6%)^D	12 (40.0%)^AD	0 (0.0%)*
Culture Positive Sepsis *n* (%)	15 (45.5%)*	3 (6.8%)^A	1 (3.3%)^A	0 (0.0%)^A
Intraventricular Haemorrhage *n* (%)	8 (24.2%)^CD	5 (11.4%)	0 (0.0%)^A	0 (0.0%)^A
Patent Ductus Arteriosus *n* (%)	19 (51.6%)*	2 (4.5%)^A	0 (0.0%)^A	0 (0.0%)^A

*p<0.05 versus every other group; ^p<0.05 versus group A, B, C or D as indicated by the corresponding letters.

Table 2: Medications that were administered to neonates during the early postnatal period.

	Group A ≤ 28 weeks (n=33)	**Group B 29 – 31 weeks (n=44)**	**Group C 32 – 36 weeks (n=30)**	**Group D ≥ 37 weeks (n=22)**
Antibiotics				
Beta-Lactam (Benzylpenicillin, Imipenem, Ampicillin) ***n* (%)**	33 (100%)^CD	44 (100%)^CD	24 (80.0%)*	0 (0.0%)*
Aminoglycoside (Gentamicin) ***n* (%)**	33 (100%)^CD	44 (100%)^CD	24 (80.0%)*	0 (0.0%)*
Glycopeptide (Vancomycin) ***n* (%)**	19 (57.6 %)*	7 (15.9%)^A	3 (10.0%)^A	0 (0.0%)^A
Macrolide (Erythromycin) ***n* (%)**	4 (12.1%)	1 (2.3%)	1 (3.3%)	0 (0.0%)
Cephalosporin (Cefotaxime, Cefozolin) ***n*(%)**	4 (12.1%)	1 (2.3%)	1 (3.3%)	0 (0.0%)
Nitroimidazole (Metronidazole) ***n* (%)**	1 (3.0%)	0 (0.0%)	1 (3.3%)	0 (0.0%)
Other				
Antifungal (Nilstat, Nystatin, Fluconazole) ***n* (%)**	18 (54.5%)^CD	17 (38.6%)^CD	4 (13.3%)^AB	0 (0.0%)^AB
Methylxanthine (Aminophylline, Theophylline) ***n* (%)**	12 (36.4%)^CD	12 (27.3%)^D	5 (16.7%)^A	0 (0.0%)^AB
Inotrope (Dopamine, Dobutamine) ***n* (%)**	9 (27.3%)*	0 (0.0%)^A	1 (3.3%)^A	0 (0.0%)^A
Steroid (Hydrocortisone) ***n* (%)**	1 (3.0%)*	0 (0.0%)^A	0 (0.0%)^A	0 (0.0%)^A
Diuretic (Furosemide) ***n* (%)**	12 (36.4%)*	3 (6.8%)^A	1 (3.3%)^A	0 (0.0%)^A
NSAID (Indomethacin) ***n* (%)**	11 (33.3%)*	1 (2.3%)^A	0 (0.0%)^A	0 (0.0%)^A

*p<0.05 versus every other group; ^p<0.05 versus group A, B, C or D as indicated by the corresponding letters.

Neonatal renal function

Urine output

Urine output adjusted for body weight (ml/kg/h) is shown in Figure 1(A-C). There was no overall effect of gestational age at birth or postnatal age on urine output in preterm neonates from postnatal days 3 to 7 (Figure 1A); urine output in all preterm neonates averaged 3.84 ± 1.3 ml/kg/h during the first week of life. On postnatal days 14 to 28, however, urine output was significantly greater in neonates with increased gestational age at birth (Figure 1B). In a comparison of urine output in preterm (Groups A, B and C) and term (Group D) neonates at postnatal day 3 and day 28 of life, there was a significant effect of both gestational and postnatal age on urine output (Figure 1C); urine output was significantly lower in term neonates compared to preterm neonates at postnatal day 3, however there was no difference between individual groups at postnatal day 28. Urine output was < 1 ml/kg/h (indicative of oliguria) in 1/44 (2%) neonates in group B (postnatal day 5) and 10/22 (45.5%) neonates in group D (postnatal day 3).

Creatinine clearance

As shown in Figure 1(D-F), in the first week of life C_{Cr} significantly increased with increasing gestational age at birth, and with increasing postnatal age (Figure 1D). Similarly, from postnatal day 14 to 28, there was also a significant effect of both gestational and postnatal age (Figure 1E); preterm neonates in the older gestational age groups had the highest creatinine clearance throughout the study period. At postnatal day 3 (Figure 1F), creatinine clearance significantly increased according to gestational age at birth. At postnatal day 28, however, creatinine clearance levels were very similar in all preterm neonates (Groups A, B and C), but significantly greater in neonates born at term (Group D). Linear regression analyses showed that there was no significant difference in the rate of change in C_{Cr} from postnatal day 3 to postnatal day 28 among the four gestational age groups: C_{Cr} increased for each week of postnatal age by 0.4 ± 0.1 ml/min/BSA in Group A, 0.4 ± 0.0 ml/min/BSA in Group B, 0.3 ± 0.1 in Group C, and 0.5 ± 0.1 ml/min/BSA in Group D. .

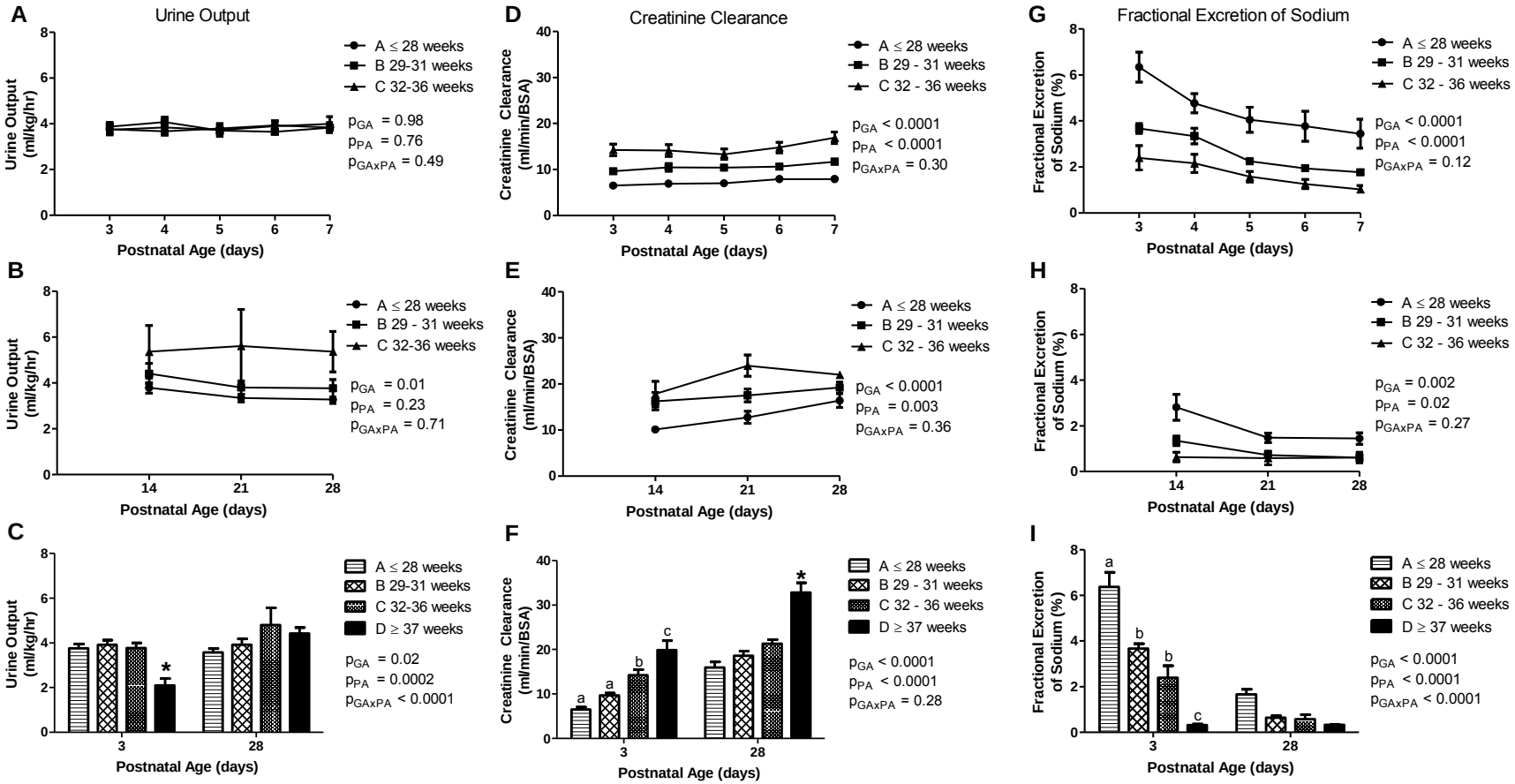

Figure 1: Urine output (A-C), the fractional excretion of sodium (D-F), and creatinine clearance (G-I) on postnatal days 3 to 7 (top row) days 14 to 28 (centre row) and day 3 versus day 28 (bottom row) in neonates born at ≤ 28 weeks gestation (Group A), 29-31 weeks gestation (Group B), 32-36 weeks gestation (Group C) and ≥ 37 weeks gestation (Group D). Data were analysed using a two-way ANOVA with the factors gestational age (p_{GA}), postnatal age (p_{PA}) and their interaction (p_{GAxPA}). From Bonferroni post-hoc analysis: *p<0.01 compared to Groups A, B and C (C and I), p<0.05 a versus b and c, b versus c (F and I).

Fractional excretion of sodium

As shown in Figure 1(G-I), the FE_{Na} significantly decreased with increasing gestational age at birth, at both postnatal days 3-7 (Figure 1G) and days 14-28 (Figure 1H). The FE_{Na} also decreased with increasing postnatal age; the highest levels of sodium excretion were observed in the extremely preterm neonates (Group A) throughout the study period. At postnatal day 3 (Figure 1I) FE_{Na} was significantly lower in the term neonates (Group D) than all other groups. By postnatal day 28, however, there was no significant difference in FE_{Na} among groups (Figure 1I). Linear regression analysis showed that the rate of change of FE_{Na} from postnatal day 3 to day 28 was significantly greater in Group B neonates compared to Group D ($p<0.0001$), and was also significantly greater in Group A neonates compared to all other groups ($p\leq0.02$). The FE_{Na} per week of postnatal age decreased by: $189 \pm 0.3 \times 10^{-3}$ percentage points in Group A, $122 \pm 0.1 \times 10^{-3}$ percentage points in Group B, and $73 \pm 0.4 \times 10^{-3}$ percentage points in Group C; whereas, the FE_{Na} remained stable in Group D).

Urine total protein, albumin, and β2-microglobulin

As shown in Figure 2, UTP, albumin and β2-microglobulin levels in preterm neonates (corrected for urine creatinine) all significantly decreased with increasing gestational age at birth. There was no change in urine protein levels with increasing postnatal age from day 7 to 28 of life. There was wide variation in urine protein levels between individual neonates within each gestational age grouping; within Group A for example, UTP:Cr levels ranged from 92.2 mg/mmol to 759.3 mg/mmol at postnatal day 28 (Figure 2A). In the term neonates (assessed only at postnatal day 3) UTP:Cr (mean: 219.3 ± 139.9 mg/mmol) and albumin:Cr (mean: 19.9 ± 9.1 mg/mmol) levels were similar to the results from neonates in Group B at postnatal day 7; β2-M:Cr levels (≤ 0.14 mg/mmol), however, were negligible. Pathological proteinuria (UTP ≥ 500 mg/L) was observed in 12 (9.3%) neonates at one or more postnatal time points, with the majority of these in the earliest gestational age grouping: Group A: n=7 (1/7 day 7, 1/7 day 14, 1/7 day 21, 3/7 day 28, and 1/7 at days 21 and 28); Group B: n=2 (1/2 day 21, and 1/2 at days 14, 21 and 28); Group C: n=1 (day 7); Group D: n=2 (day 3).

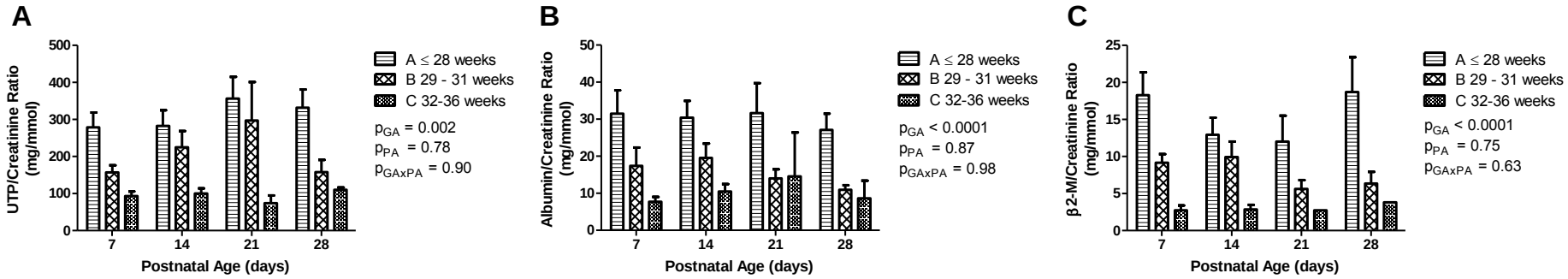

Figure 2: Urine total protein (UTP; **A**), albumin (**B**) and β2-microglobulin (β2-M; **C**) levels corrected for urine creatinine, on postnatal days 7, 14, 21, and 28 in preterm neonates (Groups A, B and C). Data were analysed using a two-way ANOVA with the factors gestational age (p_{GA}), postnatal age (p_{PA}), and their interaction (p_{GAxPA}).

Urinary NGAL

There was a significant effect of both gestational and postnatal age (p_{GA}=0.002, p_{PA}=0.02, p_{GAxPA}=0.72) on urinary NGAL, with the lowest NGAL levels observed at postnatal day 28 and in neonates at older gestational age groups. Within each age group, there was a high variability between individual neonates; for example, urinary NGAL levels ranged from 3.8 ng/ml to ≥ 500 ng/ml in the Group A neonates at postnatal day 28. Nine of the total 129 neonates (7.0%) had levels of urinary NGAL > 390 ng/ml (Group A: n=5 (2/5 postnatal day 3, 3/5 postnatal day 28); Group B: n=3 (3/3 postnatal day 28); Group C: n=1 (postnatal day 28); Group D: n=0).

Although urinary NGAL levels were not correlated with C_{Cr}, FE_{Na}, urine albumin or β-2 microglobulin levels (data not shown), there was a statistically significant linear correlation between urinary NGAL levels and UTP:Cr (Figure 3A). Importantly, however, urinary NGAL levels in the subset of preterm neonates whom exhibited pathological proteinuria (UTP ≥ 500 mg/L) were not different to the NGAL levels of age and sex-matched controls with low protein excretion at corresponding postnatal time points (Figure 3B). In these two groups, the results also indicated that urinary NGAL levels were not predictive of proteinuria, with no effect of the timing of the urinary NGAL sample (urinary NGAL levels were assessed in neonates at the time points prior to, and at the onset of, pathological proteinuria; Figure 3C). To be noted, 3 neonates with pathological proteinuria also exhibited NGAL levels ≥ 500 ng/ml; two neonates in Group A concurrently exhibited both pathological proteinuria, and high urinary NGAL levels (1/2 at postnatal day 14, 1/2 at postnatal day 28) (Figure 3B). One of these neonates also had NGAL levels ≥ 500 ng/ml at the time point prior to the onset of proteinuria (day 21), as did an additional neonate (Group A, day 21) (Figure 3C).

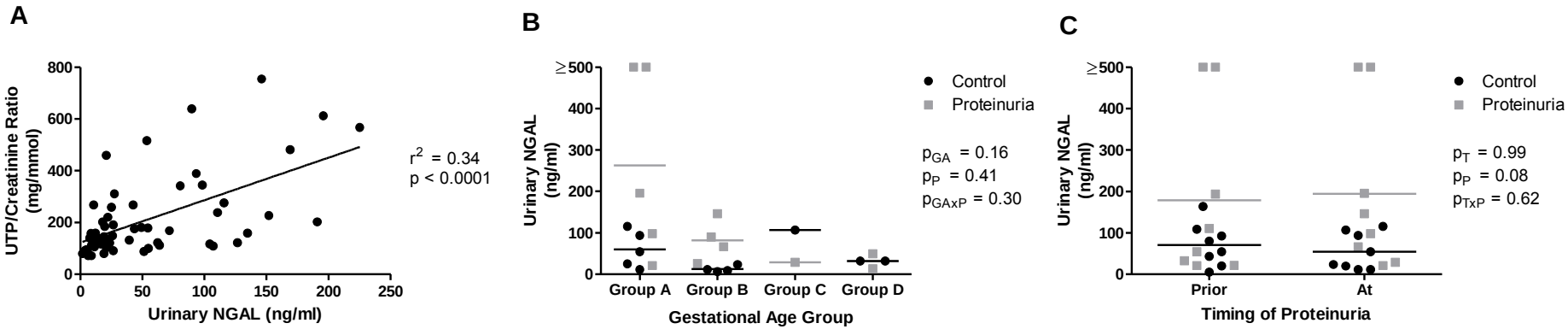

Figure 3: Linear regression analysis of urinary NGAL (< 390 ng/ml) *versus* UTP/Creatinine ratio (**A**). Urinary NGAL (ng/ml) levels were also assessed in preterm neonates with pathological proteinuria (UTP ≥ 500 mg/L; grey), with values compared to age and sex-matched controls with normal UTP at the corresponding time point (black), grouped by gestational age (**B**). Groups were compared using a two-way ANOVA, with the factors gestational age (p_{GA}), proteinuria (p_P) and their interaction (p_{GAxP}). To determine whether NGAL is predictive of proteinuria (**C**), urinary NGAL levels were assessed in the preterm neonates with pathological proteinuria (grey) compared to age- and sex-matched controls (black) at time points prior to, and at the time of, pathological proteinuria onset. The factors assessed were the time point of assessment (p_T), proteinuria (p_P) and their interaction (P_{TxP}).

Impaired Renal Function

The percentage of preterm neonates categorised as having impaired renal function (either a low urine output, low creatinine clearance, high serum creatinine, high fractional excretion of sodium, high urine total protein or high urinary NGAL, defined as values greater than 2 SD from the mean) are given in Figure 4. Overall, a similar percentage (approximately 14%) of preterm neonates in each of the gestational age groups had low urine output. High FE_{Na} levels, however, were predominantly observed in neonates born at ≤ 28 weeks gestation (Group A), with 3 preterm neonates (2/33 (6.1%) Group A, 1/44 (2.3%) Group B) exhibiting hyponatraemia (serum sodium levels < 130 mmol/L) during the study period. High UTP levels were only seen in Group B and Group C neonates, whereas high urinary NGAL levels were observed in a small percentage of neonates in each gestational age group. Twenty-five percent of neonates in Group A, 9.5% in Group B, and 7.1% in Group C had high serum creatinine levels (> 100.6 µmol/L); only one neonate (Group B), however, was categorised as having low creatinine clearance (Figure 4).

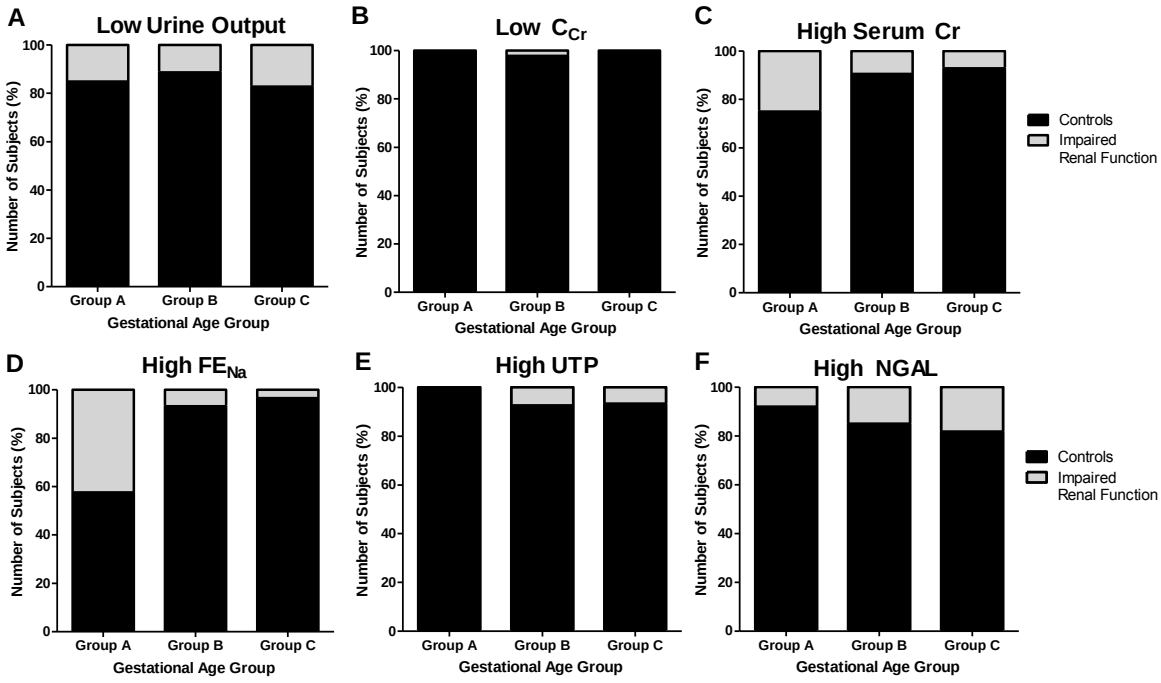

Figure 4: The percentage of neonates with impaired renal function (grey) and those with adequate renal function (black) grouped by gestational age. Impaired renal function (values more than 2 SD from the mean) was indicated by low urine output (**A**), low creatinine clearance (**B**), high serum creatinine (**C**), high fractional excretion of sodium (**D**), high urine total protein (**E**) or high urinary NGAL (**F**).

DISCUSSION

The findings of this study demonstrate that glomerular and tubular function are significantly affected by gestational age at birth; postnatal age also directly influenced renal function in preterm neonates during the first month of life. Together, these results suggest that neonatal renal function is predominantly influenced by renal structural maturity. Additionally, urinary NGAL levels did not appear to be a useful marker or predictor of acute kidney injury in this population, and thus may better reflect renal immaturity. Of concern, impairments of glomerular and tubular function were clearly evident, with overt proteinuria (suggestive of acute kidney injury) present in some of the preterm neonates. These findings highlight the importance of the close monitoring of renal function in preterm infants in the neonatal intensive care unit (NICU) setting.

Urine output

In this study, among preterm neonates urine output corrected for body weight between all gestational age groups was very similar during the first week of life. This finding was expected as within the NICU fluid intake is strictly maintained according to body weights of the neonates, and from approximately postnatal day 4 urine output in the preterm neonate is known to be predominantly influenced by fluid intake (35, 36). From days 14-28, a significantly higher urine output was observed among neonates born at older gestational ages, which likely relates to the change in feeding regimen; during this time, these neonates were either transferred to special care nurseries or discharged home where fluid intake is less controlled. In term neonates, urine output was very low at postnatal day 3 of life, with just under half of the infants exhibiting oliguria (urine output < 1 ml/kg/h). All of the term neonates were exclusively breast-fed in the first week after birth, hence the observed low urine output is likely due to low milk production; with an average of just 30 ml of maternal colostrum produced per day within the first 30-40 hours after delivery, mild dehydration is a normal occurrence which is generally well-tolerated in the term neonate (43). Importantly, during the first month of life just one preterm neonate (Group B) presented with oliguria.

Glomerular function

Creatinine clearance (C_{Cr}; an estimate of GFR that has been shown to be strongly correlated with inulin clearance (56, 64)) was significantly affected by both gestational age at birth and postnatal age in preterm neonates; similar findings have been previously reported (3, 7, 11, 12, 14, 17, 19, 29, 48, 57, 61, 67). On day 3 of life, C_{Cr} was significantly lower among those preterm neonates born at < 31 weeks of gestation (Groups A and B) compared to the moderately preterm and term neonates. By postnatal day 28, C_{Cr} was not significantly different between the three gestational age groups of preterm neonates, which suggests that although neonates born very or extremely preterm commence with a low GFR during early life, they have caught up to those born moderately preterm by day 28 of life. As expected, in comparison to term neonates C_{Cr} was significantly lower in all preterm neonates at postnatal day 28. Interestingly, however, the rate of change in C_{Cr} over the first month of life was very similar between all groups, and we did not observe the slower postnatal increase in C_{Cr} in preterm neonates compared to term controls that has been reported previously (7, 19). Overall, these results suggest that renal functional capacity increases with renal structural maturity, potentially through increased filtration surface area (increased number of nephrons in those neonates with ongoing postnatal nephrogenesis (58) and/or increased capillary growth (40, 58)) as well as the substantial changes in renal blood flow and renal vascular resistance that occur after birth (65).

In the present study, 25% of neonates in Group A, and less than 10% in Groups B and C, exhibited high serum creatinine levels; there was just one preterm neonate (Group B), however, that had C_{Cr} levels < 2 SD from the mean (from day 3-6 of life). Estimates of GFR (such as C_{Cr}) are generally considered to be a more accurate measure of glomerular function than serum creatinine alone, although it is to be noted that the potential for active tubular secretion of creatinine, as well as differences in muscle mass between neonates, may affect the accuracy of the results (55). Importantly, a low C_{Cr} is likely to impair the renal clearance of nephrotoxic medications that may adversely affect the developing kidney (52). In this regard, the individual neonate with low C_{Cr} also exhibited pathological proteinuria (UTP ≥ 500 mg/L on postnatal days 14, 21 and 28), however the cause of the severe proteinuria is unknown.

The glomerular filtration barrier is responsible for preventing the passage of high molecular weight proteins (such as albumin) from entering the filtrate (23); therefore, urine albumin (and also UTP) levels are important indicators of glomerular function. In accordance with the findings of previous studies (6, 11, 15, 16, 62), we observed that urine albumin and UTP levels were significantly higher in neonates with a low gestational age at birth. There was no change, however, in urine protein levels with increasing postnatal age over the first month of life. These results may suggest that the observed proteinuria is due to structural immaturity of the glomerular filtration barrier, which persists until at least one month of age. Alternatively, these findings may be indicative of renal injury in the preterm neonate.

In this regard, UTP levels were highly variable between individual neonates, and 12 neonates in particular had pathological proteinuria at one or more postnatal time point. Interestingly, the onset of this severe proteinuria was often observed at postnatal days 21 and 28 (7/12 (58%) of neonates that exhibited pathological proteinuria), which suggests that factors in the postnatal clinical course of the preterm neonate (rather than renal immaturity) may be the cause of the severe proteinuria. In support of this idea, it was only neonates born at older gestational ages (Groups B and C) that exhibited high UTP levels > 2 SD from the mean. In this regard, exposure to nephrotoxic medications (including NSAIDs and antibiotics) is known to cause renal injury, such as the effacement of podocyte foot processes (30), and they may also impair nephrogenesis (52, 59). In this study, due to the many confounding factors associated with the clinical care of the preterm neonate, it was not possible to identify the factors contributing to the severe proteinuria in the preterm neonates; for such an analysis, a very large sample size would be required. A better approach may be to use carefully controlled animal studies where the effects of specific factors can be individually addressed.

Importantly, in the short term it is conceivable that excessive protein excretion may impact on the nutritional requirements of the neonate, and thus adversely affect postnatal growth (34). Furthermore, the presence of persistent proteinuria is likely to lead to long-term renal impairment. Indeed, proteinuria has been linked to progressive renal damage (1, 42), and renal injury in the neonatal period may have long-term adverse effects on renal function (2).

Tubular function

It is essential that a positive sodium balance is achieved in the neonate in order to support postnatal growth and development (25); measurements of the FE_{Na} were made in the present study to assess the reabsorptive capacity of the renal tubule. Consistent with previous studies (3, 17, 18, 48, 57), we found a significant effect of both gestational and postnatal age on the FE_{Na} in preterm neonates during the first month of life, with the highest FE_{Na} levels observed in the preterm neonates born extremely preterm (≤ 28 weeks gestation). A relatively high percentage of neonates, predominantly those born extremely preterm, were observed over the duration of the study to have FE_{Na} levels > 2 SD from the mean. The high level of sodium excretion in these neonates leads to an elevated risk of hyponatraemia (39); however, likely due to the administration of sodium supplementation in this population, just three preterm neonates (Group A and B) had serum sodium levels < 130 mmol/L during the study period.

Unlike the persistently low C_{Cr} observed in preterm neonates during the first month of life, there was no significant difference in FE_{Na} levels among preterm and term neonates by postnatal day 28, indicating a high capacity for postnatal tubular maturation. This was particularly evident in the neonates born ≤ 28 weeks gestation (Group A) where there was a significantly greater rate of change in FE_{Na} from postnatal day 3 to day 28 compared to all other groups. The maturation of tubular function with increasing age is known to be mediated by increases in the basolateral surface area of tubular cells (4, 13), increased activity of sodium transporter proteins (5, 21), and the action of multiple endocrine systems (9, 50). Indeed, by the time of term birth the ability of the kidney to conserve sodium (despite the relatively low sodium content of human breast milk (31)) has been shown to be very strong (9, 50).

The presence of low molecular weight proteins in the urine is also an important indicator of an impairment of tubular function, as these proteins are usually endocytosed (via megalin and cubilin receptors) within the proximal tubule after passing unrestricted through the glomerular filtration barrier (10). As observed in prior studies (3, 62, 63), and consistent with the urine albumin and urine total protein results, β2-microglobulin levels were highest in neonates with a low gestational age at birth, and there was no change

over the first month of life. Unlike the significant reduction in sodium excretion with increasing maturity, the capacity for protein reabsorption remained low postnatally which possibly reflects a much slower maturation of renal protein handling compared to sodium handling; although qualitative expression of megalin/cubilin throughout renal development has been previously described (49), to our knowledge quantitative changes in expression levels with increasing gestational age have not been reported. Alternatively, it is conceivable that proximal tubule cell injury (such as occurs following oxidative stress in the preterm neonate (46)), and/or an overload of filtered protein (given the protein fortification of milk that is essential for adequate postnatal growth in the preterm neonate (38)) may have additionally impacted on tubular protein uptake.

Urinary NGAL as a marker of acute kidney injury?

NGAL is excreted by renal proximal tubule cells as a response to acute kidney injury (51); however, NGAL is also known to be produced during nephrogenesis (22), and levels may also be raised due to unrelated clinical conditions such as late-onset sepsis (45). Although there have been very promising results in populations of older paediatric patients (68), the usefulness of urinary NGAL as a marker of AKI in preterm neonates remains unclear (28, 32, 44, 45). Certainly, in the present study we found a significant correlation between urinary NGAL levels and both gestational and postnatal age, which is consistent with previous studies (28, 32); these findings likely relate to the immaturity of the kidney, and the clinical instability of the younger neonates. Importantly, the results of the current study demonstrated that urinary NGAL levels were not directly correlated with any indicators of renal function, apart from UTP. Although two preterm neonates were observed to have concurrently high UTP and NGAL levels, which are suggestive of significant renal injury in these individual neonates, in general the neonates with pathological proteinuria did not exhibit high urinary NGAL levels. There was also no association between NGAL levels at time points before or at the time of proteinuria onset, indicating that NGAL may not be predictive of renal injury. As such, the clinical utility of NGAL in the diagnosis of acute kidney injury in the preterm neonate is questionable.

Strengths and limitations of the study

This is one of only a few studies to have examined renal function among a large sample of preterm babies during the first month of life. A wide variety of measures were used in order to comprehensively assess both glomerular and tubular function, including levels of urinary protein which has been examined in only a limited number of previous studies. In this regard, one of the limitations of the study was the difficulties in obtaining spot urine samples in order to undertake the protein analyses. Overall, 46.2% of the total number of requested urine samples were obtained. Given the immaturity of the preterm kidney it is important to determine what levels of protein in the urine are normal, *versus* those that are pathological and thus indicative of renal injury in preterm neonates. In order to do this, it is firstly important to establish the normal range of urinary protein levels in preterm neonates. The results from this study have been important in working towards the development of a normal range; however, our findings are limited by the relatively small sample size.

Conclusions: The findings of this study demonstrate that renal maturity is the most important determinant of glomerular and tubular function among preterm neonates, with both C_{Cr} and FE_{Na} improving significantly in all gestational age groups by day 28 of life. Of particular concern, a number of preterm neonates exhibited severe proteinuria which is likely indicative of postnatal renal injury; urinary NGAL, however, was not a useful marker or predictor of renal injury in this population. In future studies it is important to identify the specific factors in the postnatal clinical care of the preterm neonate which may be leading to the high urinary protein excretion. The consequences of proteinuria in the neonatal period are unknown; however, the potential for progressive renal injury and long-term renal dysfunction suggest the need for regular assessments of renal function in subjects that are born preterm.

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CHAPTER THREE

ACCELERATED MATURATION AND ABNORMAL MORPHOLOGY IN THE PRETERM NEONATAL KIDNEY

CHAPTER THREE DECLARATION

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

Nature of contribution	Extent of contribution (%)
Performed half of experimental work, majority of data analysis, and co-authored manuscript	50%

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution (%) For student co-authors only
Lina Gubhaju	Performed half of experimental work, some data analysis, co-authored manuscript	40%
Lynette Moore, Alison Kent, Jane Dahlstrom, Rosemary Horne, Wendy Hoy, John Bertram, M. Jane Black	Involved in the collection of kidney tissue, provided medical and autopsy records, and assisted in editing the manuscript	10%

Candidate's Signature [redacted] **Date** 30/04/12

Declaration by co-authors

The undersigned hereby certify that:

(25) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(26) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(27) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(28) there are no other authors of the publication according to these criteria;
(29) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(30) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) **Department of Anatomy & Developmental Biology, Monash University**

	Signature	Date		Signature	Date
Lina Gubhaju	[redacted]	24/4/2012	**Wendy Hoy**	[redacted]	24/4/12
Lynette Moore		30-4-12	**John Bertram**		26/4/12
Alison Kent		24.4.12	**M. Jane Black**		24/4/12
Jane Dahlstrom		24/4/12			
Rosemary Horne		24/4/12			

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Accelerated Maturation and Abnormal Morphology in the Preterm Neonatal Kidney

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ABSTRACT

Nephrogenesis is ongoing at the time of birth for the majority of preterm infants, but whether postnatal renal development follows a similar trajectory to normal *in utero* growth is unknown. Here, we examined tissue collected at autopsy from 28 kidneys from preterm neonates, whose postnatal survival ranged from 2 to 68 days, including 6 that had restricted intrauterine growth. In addition, we examined kidneys from 32 still-born gestational controls. We assessed the width of the nephrogenic zone, number of glomerular generations, cross-sectional area of the renal corpuscle, and glomerular maturity and morphology. Renal maturation accelerated after preterm birth, with an increased number of glomerular generations and a decreased width of the nephrogenic zone in the kidneys of preterm neonates. Of particular concern, compared with gestational controls, preterm kidneys had a greater percentage of morphologically abnormal glomeruli and a significantly larger cross-sectional area of the renal corpuscle, suggestive of renal hyperfiltration. These observations suggest that the preterm kidney may have fewer functional nephrons, thereby increasing vulnerability to impaired renal function in both the early postnatal period and later in life.

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The incidence of preterm birth continues to be high in many countries worldwide.[1,2] Furthermore, the survival of preterm neonates, particularly those born extremely preterm, has improved substantially over recent decades with those born as early as 25 weeks of gestation reported to have an 82% chance of survival.[3] Preterm birth, however, is associated with many postnatal complications during the neonatal period primarily because of the vulnerability of the immature organs.[4,5] In this regard, renal dysfunction is commonly observed in preterm neonates.[6–8] and this is likely to be associated with the immaturity of the kidney at the time of birth. Nephrogenesis in the human kidney does not reach completion until approximately 36 weeks of gestation, with the majority of nephrons formed in late gestation at a time when preterm infants have already been delivered.[9,10]

The immature preterm kidney with ongoing nephrogenesis is likely to be vulnerable to the he-

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modynamic changes associated with preterm birth. Hence, it is important to determine whether kidney development follows the normal growth trajectory of that *in utero*, after preterm delivery. To date, there have been few studies examining the effect of preterm birth on nephrogenesis.[11,12] A human autopsy study by Rodriguez *et al.*[11] found that compared with term-born infants, the number of radial glomerular counts (glomerular generations) in the kidney, an index of renal maturity, was significantly reduced in preterm infants. It is important to note, however, that a large proportion of the neonates in the preterm group in this previous study were intrauterine growth restricted (IUGR), which is a known cause of low nephron endowment.[9,13,14] A recent autopsy study by Faa *et al.*[12] also examined autopsied kidneys from 12 premature neonates and found a low number of radial glomerular counts and marked interindividual variability among preterm neonates. It could not be ascertained from the study whether any of the preterm neonates were also IUGR.

Recent nonhuman primate studies, undertaken using a baboon model of preterm birth, have demonstrated that although nephrogenesis continued after preterm delivery, postnatal nephrogenesis was associated with an increased risk of abnormal glomerular development, with the proportion of abnormal glomeruli ranging from 0.2% up to 18%.[15,16] The aim of the current study was to examine renal morphology and glomerular maturation in the preterm human neonate and to determine whether preterm birth also leads to the development of abnormal glomeruli in the human kidney.

RESULTS

Cause of Death and Illness

The most common causes of death and illness in the preterm neonates included sepsis (58%), intraventricular/intracranial hemorrhage (42%), necrotizing enterocolitis (37%), and respiratory disease (32%). There was only one neonate in the preterm group that was diagnosed with acute renal failure and one neonate with mild renal failure. Six of the preterm neonates were diagnosed with IUGR (two per postconceptional age grouping). The majority of the gestational controls investigated in this study died acutely *in utero* with an undetermined cause of death (72%). Other causes included acute asphyxia (16%), feto-maternal hemorrhage (6%), and preterm labor (6%).

Neonatal Age and Growth Characteristics (Table 1)

Gestational age at birth in the two preterm groups ranged from 24 to 35 weeks and postnatal age ranged from 2 to 68 days. Gestational age of the stillborn control neonates was in the same range as the postconceptional age of the preterm neonates (24 to 38 weeks). Gestational age of the non-IUGR and IUGR preterm neonates at birth was significantly lower compared with the gestational controls; however, postconceptional age was similar in all groups.

Total combined kidney weights were also similar between groups (Figure 1A). Kidney weight relative to body weight, however, was significantly greater in the preterm group compared with the gestational control group ($P < 0.05$) (Figure 1B). Within the two preterm groups, only birth weight was significantly smaller in the IUGR preterm neonates (Table 1); however, there was no difference in body weights at autopsy, kidney weight, or kidney-to-body weight ratio.

There was a significant positive correlation between body weight at autopsy and postconceptional age in both the preterm neonates and the gestational controls (control: $r^2 = 0.87$, $P < 0.0001$; preterm: $r^2 = 0.66$, $P < 0.0001$). Kidney weight also correlated significantly with increasing postconceptional age in both groups (control: $r^2 = 0.57$, $P < 0.0001$; preterm: $r^2 = 0.59$, $P < 0.0001$).

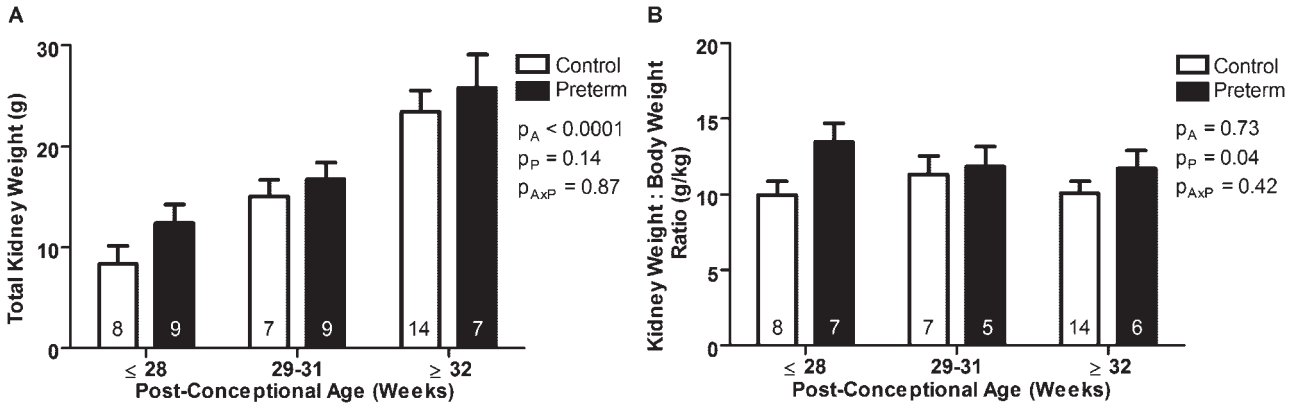

Figure 1. Increased kidney-to-body weight ratio in preterm neonates. (A) Total combined kidney weight and (B) kidney weight relative to body weight in gestational controls (white bars) and preterm neonates (black bars), grouped by postconceptional age. The number of neonates in each group is indicated on the bars. Total kidney weight significantly increased with increasing postconceptional age (A). Compared with the gestational controls, the preterm neonates had a significantly increased kidney-to-body weight ratio (B). Bars represent mean ± SEM. *P*A, postconceptional age; *P*P, prematurity; *P*AxP, interaction.

Table 1. Age, sex, and body weights of the gestational controls and preterm neonates (non-IUGR and IUGR)

	Control (n = 32)	Preterm (n = 22)	Preterm + IUGR (n = 6)
Gestational age (weeks)	31.0 ± 0.8 (24 to 38)	27.9 ± 0.7* (24 to 35)	27.0 ± 0.7* (25 to 30)
Postnatal age (days)		18.3 ± 3.4 (2 to 42)	30.7 ± 12.5 (2 to 68)
Postconceptional age (weeks)	31.0 ± 0.8 (24 to 38)	30.0 ± 0.7 (24 to 38)	31.4 ± 1.8 (25 to 37)
Sex ratio (M:F)	16:16	13:9	3:3
Birth weight (g)	1619 ± 147	1228 ± 107	628 ± 108*#
Autopsy weight (g)	1642 ± 146	1468 ± 157	1150 ± 330

Data presented as mean ± SEM with data range in parentheses. *$P < 0.05$ compared with control group; #$P < 0.05$ compared with preterm group.

Assessment of Nephrogenesis

Nephrogenic Zone Width.

In 10 kidneys (4 preterm and 6 gestational controls with a postconceptional age ≥32-weeks) nephrogenesis was complete before autopsy, with no evidence of a nephrogenic zone. In kidneys with ongoing nephrogenesis the width of the nephrogenic zone tended to decrease with increasing postconceptional age ($P = 0.06$) (Figure 2A). Overall, nephrogenic zone width was significantly decreased in the preterm group compared with the gestational controls ($P < 0.001$).

Glomerular Generations.

Kidneys with ongoing nephrogenesis and those with completed nephrogenesis were analyzed separately. In kidneys that had completed nephrogenesis (6 controls and 4 preterm), there was no significant difference in the average number of glomerular generations between the preterm group compared with the gestational controls (control: 7.8 ± 0.4; preterm: 8.3 ± 0.6; $P = 0.55$). In kidneys with ongoing nephrogenesis, the number of glomerular generations increased significantly with postconceptional age (Figure 2B). Importantly, the number of glomerular generations formed was significantly greater in the preterm kidneys compared with the gestational controls ($P < 0.05$).

There was a significant positive correlation between body weight at autopsy and the number of glomerular generations in both the preterm group ($r^2 = 0.54$, $P < 0.001$) and the gestational controls ($r^2 = 0.54$, $P < 0.0001$). Similarly, there was a significant positive correlation between kidney weight and the number of glomerular generations within both groups (control: $r^2 = 0.42$, $P < 0.001$; preterm: $r^2 = 0.43$, $P < 0.001$).

Assessment of Glomerular Size, Maturity, and Morphology

Renal Corpuscle Cross-sectional Area.

Average renal corpuscle cross-sectional area was significantly greater in the preterm group than in the gestational controls ($P < 0.0001$) (Figure 2C). Linear regression analyses showed no correlation between mean renal corpuscle area and gestational age, postconceptional age, or postnatal age in either the control or the preterm groups.

Glomerular Maturity.

The percentage of glomeruli in the immature stages of glomerular development (stages V [vesicle], S [comma-shaped and S-shaped], and C [capillary loop]) significantly decreased with increasing postconceptional age ($P < 0.01$) (Figure 3). Conversely, the percentage of glomeruli at stages II and III of development significantly increased with increasing postconceptional age ($P < 0.01$). The most common stage of development, with approximately 50% of all glomeruli in each

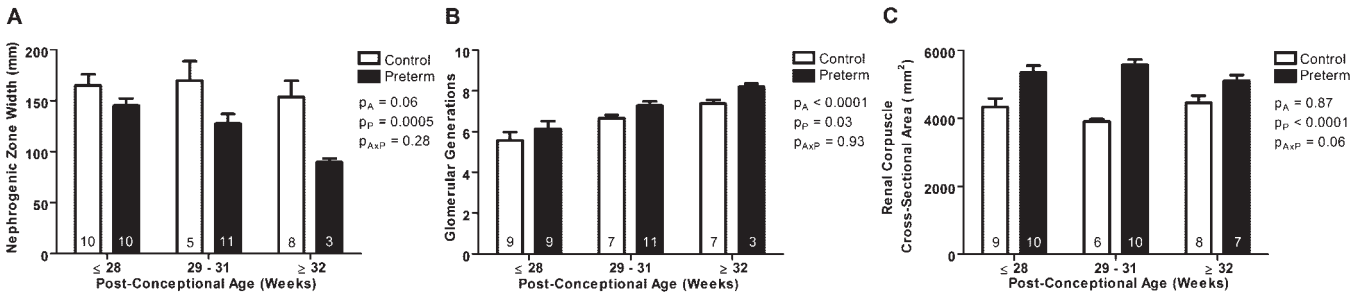

Figure 2. Decreased nephrogenic zone width, increased number of glomerular generations, and increased renal corpuscle size in kidneys from preterm neonates. (A) Nephrogenic zone width, (B) glomerular generation number, and (C) mean renal corpuscle cross-sectional area in kidneys from gestational controls (white bars; 6 neonates with completed nephrogenesis were not included in A and B) and preterm neonates (black bars; 4 neonates with completed nephrogenesis were not included in A and B), grouped by postconceptional age. The number of neonates in each group is indicated on the bars. Average nephrogenic zone width was significantly less in preterm neonates compared with gestational controls (A). The number of glomerular generations significantly increased with increasing postconceptional age, and was significantly greater in the preterm neonates compared with the gestational controls (B). Mean renal corpuscle cross-sectional area was significantly larger in the preterm neonates compared with the gestational controls (C). Bars represent mean ± SEM. *P*A, postconceptional age; *P*P, prematurity; *P*AxP, interaction.

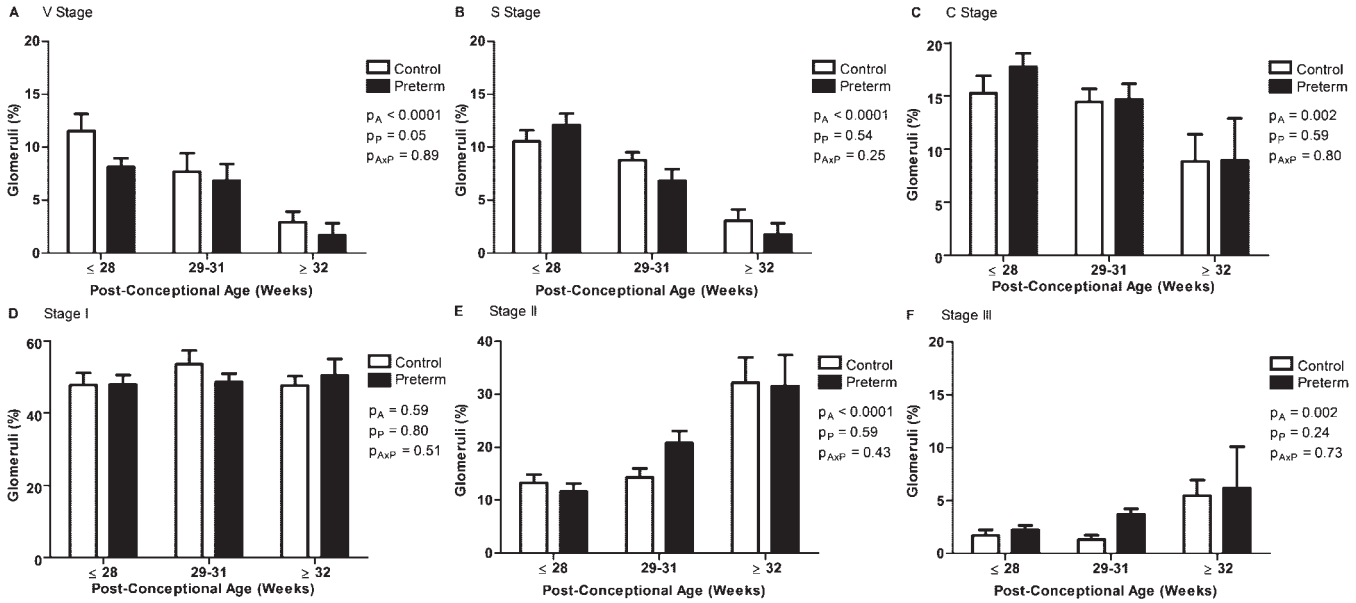

Figure 3. Percentage of immature V-stage glomeruli is reduced in the kidneys of preterm neonates. The percentage of glomeruli at each stage of maturity, in the gestational control (white bars) and preterm neonates (black bars), grouped by postconceptional age. Control: ≤28 weeks (n = 9), 29 to 31 weeks (n = 5), ≥32 weeks (n = 9); preterm: ≤28 weeks (n = 8), 29 to 31 weeks (n = 12), ≥32 weeks (n = 6). The percentage of glomeruli at stages V (A), S (B), and C (C) significantly decreased with increasing postconceptional age, whereas the percentage of glomeruli at stages II (E) and III (F) significantly increased. There was no change in the percentage of glomeruli at stage I with increasing postconceptional age (D). A significantly lower percentage of V-stage glomeruli were observed in the preterm kidneys compared with the gestational controls (A). Bars represent mean ± SEM. *P*A, postconceptional age; *P*P, prematurity; *P*AxP, interaction.

kidney, was stage I; there was no change in the percentage of glomeruli at stage I with increasing postconceptional age. Importantly, the percentage of immature V-stage glomeruli was significantly less in the preterm group than in the gestational controls ($P \leq 0.05$).

Glomerular Morphology.

Morphologically abnormal glomeruli, with a dilated Bowman's space and shrunken tuft, were commonly observed in the outer cortex of preterm kidneys (Figure 4). All abnormal glomeruli were noted to be in stage I of development. Overall, the proportion of abnormal glomeruli was significantly greater in the kidneys of the preterm neonates compared with the gestational controls (Figure 5). In preterm kidneys, the proportion of abnormal glomeruli ranged from 0 to 13.7%, with a similar range observed across all postconceptional age groupings. The kidney with the highest proportion of abnormal glomeruli (13.7%) was from a preterm neonate diagnosed with IUGR. The percentage of abnormal glomeruli did not correlate with gestational, postconceptional, or postnatal age.

Neonatal Medications

The full medical history, including the administration of medications, was available for 20 of the 28 preterm neonates. The most commonly administered medications were antenatal steroids (55%), antibiotics (80%), nonsteroidal anti-inflammatory drugs (indomethacin) (50%), and inotropes (dopamine and dobutamine) (80%). Exposure to prenatal and/or postnatal medications was not associated with an increased percentage of abnormal glomeruli.

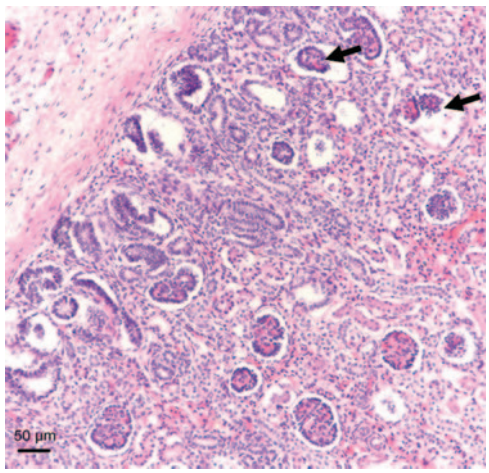

Figure 4. Abnormal glomerular morphology in the kidney of a preterm neonate. Representative photomicrograph depicting abnormal glomeruli, with dilated Bowman's space and shrunken tuft (arrows), in the outer cortex of a preterm neonatal kidney.

Oligonephronia in a Preterm Neonate

One preterm neonate was excluded from all analysis because of significant renal abnormalities. This neonate, a male born at 28

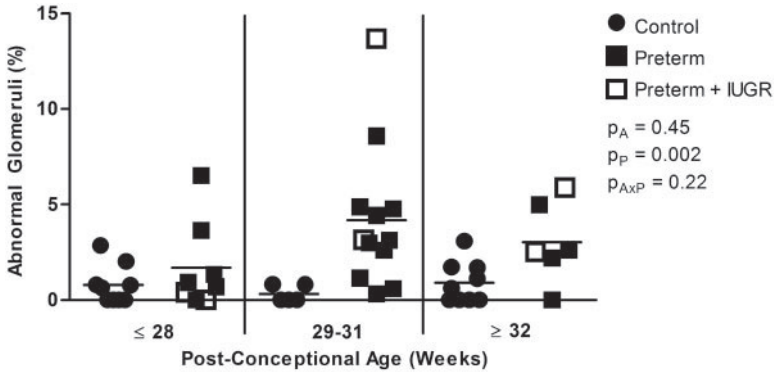

Figure 5. Increased percentage of morphologically abnormal glomeruli in kidneys from preterm neonates. The percentage of abnormal glomeruli in the kidneys of gestational controls and preterm neonates, grouped by postconceptional age. Control: ≤28 weeks (n = 9), 29 to 31 weeks (n = 5), ≥32 weeks (n = 9); preterm: ≤28 weeks (n = 8), 29 to 31 weeks (n = 12), ≥32 weeks (n = 6). The percentage of abnormal glomeruli was significantly increased in the preterm group compared with the gestational controls. *P*A, postconceptional age; *P*P, prematurity; *P*AxP, interaction.

weeks gestation, survived postnatally for 19 days with death attributed to necrotizing enterocolitis. Preterm delivery was associated with a history of maternal pre-eclampsia and histologic evidence of grade 2 (of 3) acute chorioamnionitis. Postnatally, he was exposed to 5 doses of indomethacin and 9 doses of gentamicin.

As shown in Figure 6, this neonate's kidney had no evidence of a nephrogenic zone at the time of analysis (30.7-week postconceptional age). Ninety-two percent of glomeruli within the kidney were at stage III of development, and average renal corpuscle area was 34,184 μm^2 (approximately sixfold larger than in any other kidney). No abnormal glomeruli were observed in this kidney.

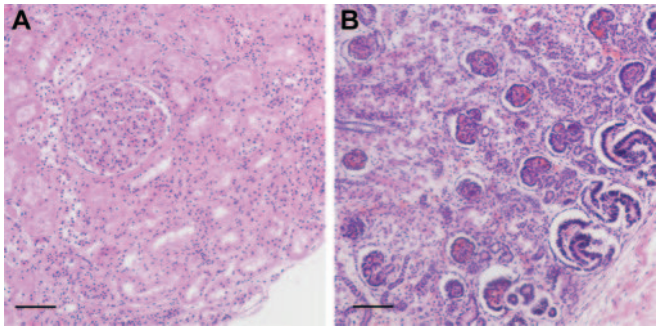

Figure 6. Larger mean renal corpuscle area in a preterm neonate with oligonephronia. Representative photomicrographs of the outer renal cortex of (A) one preterm neonate with oligonephronia (postconceptional age 30.7 weeks) and (B) one preterm neonate with the appearance of normal renal development (postconceptional age 30.0 weeks). In the neonate with oligonephronia, nephrogenesis is complete, with the single visible glomerulus at stage III of development. In contrast, the kidney of the preterm neonate in (B) exhibits an active nephrogenic zone, and the numerous outer cortical glomeruli are in S-stage, C-stage, and stage I of development. Furthermore, mean renal corpuscle area was significantly larger in the neonate with oligonephronia (A). Bar represents 100 μm.

DISCUSSION

This study comprehensively examines the effects of preterm birth on the normal growth trajectory in autopsied kidneys from both IUGR and non-IUGR preterm neonates compared with stillborn gestational controls. Through this series of structural analyses we have shown that preterm neonates exhibit accelerated postnatal renal maturation with a reduced nephrogenic zone width, reduced percentage of immature V-stage glomeruli, and an increased number of glomerular generations compared with postconceptional age-matched gestational controls. In addition, and of particular concern, preterm kidneys exhibited an enlarged renal corpuscle cross-sectional area and morphologically abnormal glomeruli, with up to 13% of glomeruli in the kidney affected. These findings may have important clinical implications for both the short- and long-term renal health of infants born preterm.

In agreement with previous studies,[16,17] the preterm neonates had a significantly increased kidney-to-body weight ratio compared with the gestational controls. Mean renal corpuscle area was also significantly increased in preterm kidneys, averaging over 5000 μm^2 compared with approximately 4000 μm^2 in controls. These results were anticipated because the neonatal kidneys have undergone a dramatic hemodynamic change after birth whereby the renal system switches from a fetal to a neonatal organ with high blood flow and low vascular resistance.[18,19] Whether the glomerular enlargement observed in the preterm kidneys is indicative of glomerular hyperfiltration, however, cannot be determined in the current study. As the full complement of nephrons is not achieved until late in gestation, it is conceivable that the preterm kidney may not be able to cope with the postnatal functional demands, resulting in compensatory glomerular hypertrophy. Previous studies have shown that glomerular hypertrophy and hyperfiltration lead to glomerular injury and later nephron loss; these detrimental changes are strongly linked to the development of long-term renal disease.[20,21] Further studies are required to ascertain whether the glomerular hypertrophy is a pathologic, or a normal physiologic, process.

Importantly, we have demonstrated that preterm birth is associated with accelerated renal maturation. Our findings suggest that nephrogenic zone width is significantly reduced in the kidneys of preterm neonates compared with gestational controls at similar postconceptional ages, suggestive of early cessation of nephrogenesis in the postnatal environment and/or accelerated maturation of glomeruli. In support of this

finding, the percentage of immature V-stage glomeruli was significantly reduced in the preterm kidney than in the gestational controls. Furthermore, glomerular generation number was significantly increased in the preterm kidneys than in gestational controls at similar postconceptional ages. The mechanisms leading to the accelerated renal maturation observed in the preterm kidney are unknown, but may relate to factors in the care of the neonate which promote organ maturation. Indeed, exposure to antenatal glucocorticoids, which are commonly administered before preterm delivery to aid postnatal respiratory function,[22] is associated with renal functional maturation in the preterm neonate.[23] Furthermore, the administration of betamethasone is reported to lead to a thinning of the nephrogenic zone in the fetal rhesus monkey[24] and an increased number of developed glomeruli were found in the kidneys from preterm baboons after antenatal exposure.[16] In this study, many of the preterm neonates were administered antenatal steroids. However, because full medical histories could not be obtained for the whole cohort, we were unable to determine if there were any maturational differences between the neonates that were exposed to steroids compared with the ones that were not.

In contrast, the results of previous autopsy studies by Rodriguez *et al.*[11] and Faa *et al.*[12] indicated a reduced number of glomerular generations in the preterm kidney compared with infants that were born at term, perhaps indicative of a nephron deficit. In a nonhuman primate model of preterm birth, however, we have previously demonstrated that nephron endowment was not affected by preterm birth.[16] Similarly, in the current study we found no difference in glomerular generation number between gestational control and preterm neonates in which nephrogenesis was complete (ranging from 7 to 10 generations in each group). Differences in the postnatal clinical course of the preterm neonates examined in the current study and those in previously published studies[11,12] may also account for the contrasting findings. Indeed, Faa *et al.*[12] observed marked interindividual variations in radial glomerular counts.

We had also initially hypothesized that the findings of a reduced number of glomerular generations in the preterm kidney by Rodriguez *et al.*[11] may have been confounded by the inclusion of preterm neonates that were also IUGR since this possibility was not explored by the authors. In the present study, although the included IUGR preterm neonates were significantly smaller than the non-IUGR preterm neonates at birth, there was no significant difference in kidney or body weights at autopsy. Hence, it is not surprising that no differences were found in the indices of renal development. This may be due to catch-up growth of the kidney postnatally after preterm birth in the IUGR neonates and/or the severity of the IUGR. Furthermore, a limitation of the current study is that only a small number of preterm IUGR neonates were assessed, which may have reduced the potential to observe statistical differences.

An important finding from this study is that many glomeruli in the outer cortex of the preterm kidney were morphologically abnormal, exhibiting an enlarged Bowman's space and shrunken glomerular tuft, and therefore unlikely to be functional. The abnormal glomeruli (previously observed in human[11] and baboon[15,16] preterm neonates) were only present in the outer cortex, suggesting that it is those glomeruli newly formed in the extrauterine environment that are "at risk". Certainly, kidneys with a large number of abnormal glomeruli are likely to suffer a significant deficit of functional nephrons, which is strongly linked to an increased susceptibility to hypertension and renal disease later in life.[25] In this regard, a number of recent studies have linked preterm birth with both the development of hypertension[26–36] and renal dysfunction;[37] perhaps one factor underlying these associations is a reduced nephron endowment in infants born preterm. Further research is needed, however, before this can be fully elucidated.

The large variation in the percentage of abnormal glomeruli between neonates in this study, as well as in our previous studies,[15,16] suggests that these abnormalities have not occurred as a result of preterm birth *per se*, but may be related to differences in the postnatal clinical course of the neonates. No clear link between postnatal medication exposure and the extent of glomerular abnormalities was found in this study or in previous studies,[15] possibly because of the low sample size. Certainly, however, the oligonephronia observed in one of the preterm neonates, who had been exposed to a large number of doses of both antibiotics (gentamicin) and nonsteroidal anti-inflammatory drugs (indomethacin) supports previous *in vivo* and *in vitro* studies in experimental models that have demonstrated adverse effects on nephrogenesis[38–40] and renal morphology.[41] In future, it is essential to identify potentially modifiable factors in the postnatal care of the preterm neonate (such as postnatal administration of antibiotics and nonsteroidal anti-inflammatory drugs) that may be associated with impaired nephrogenesis and abnormal glomerular development.

In conclusion, this study comprehensively examines postnatal nephrogenesis in the human preterm kidney. We found a decreased nephrogenic zone width, decreased percentage of immature V-stage glomeruli, and an increased number of glomerular generations, which suggests accelerated postnatal renal maturation. Of concern, there was a significant increase in renal corpuscle cross-sectional area and up to 13% of glomeruli were morphologically abnormal. Together, these detrimental changes in the immature glomeruli may ultimately result in a nephron deficit, which is linked to the development of renal disease and hypertension later in life. These findings, therefore, have significant implications for both the short- and long-term renal health of infants born preterm.

CONCISE METHODS

In this retrospective study, archived neonatal kidneys were obtained from the Women's and Children's Hospital in North Adelaide, South Australia, and The Canberra Hospital in Woden, Australian Capital Territory. Autopsies were performed in the range of years 1996

through 2009. Ethics approval for autopsy was obtained from the Children, Youth and Women's Health Service Research Ethics Committee of South Australia and the Australian Capital Territory Human Research Ethics Committee. Only infants with written informed parental consent for autopsy were included in the study.

Inclusion and Exclusion Criteria

Preterm neonates with postnatal survival >2 days were included in this study. Any neonate with evidence of a congenital abnormality was excluded. Neonates were also excluded if the kidneys were severely macerated and/or the estimated time between fetal death and delivery was >48 hours. One preterm infant was subsequently excluded because of the significant renal abnormalities observed.

Both IUGR and non-IUGR preterm neonates were included in this study; however, data were analyzed separately to identify if there was any effect of IUGR on the preterm kidney. Gestational controls diagnosed with IUGR were excluded.

Neonatal Characteristics

Twenty-eight preterm neonates with postnatal survival of 2 or more days, and 32 gestational controls were examined in this study. Six of the preterm neonates had been diagnosed with IUGR (based on birth weight, growth parameters, and brain weight-to-liver weight ratios). Gestational age at birth was estimated using both the mother's last menstrual cycle and the Dubowitz clinical characteristics.[42] Gestational controls were stillborn neonates that died acutely *in utero*.

Neonates were further divided according to postconceptional age (sum of gestational age and postnatal age in weeks): ≤28 weeks (extremely preterm; control, $n = 9$; preterm, $n = 10$), 29 to 31 weeks (very preterm; control, $n = 8$; preterm, $n = 11$), and ≥32 weeks (moderately preterm; control, $n = 15$; preterm, $n = 7$). There were two preterm IUGR neonates included in each of the postconceptional age groupings.

Clinical History

Maternal and neonatal clinical histories were obtained from the autopsy reports and hospital medical records where available; we had access to the full medical records from 20 preterm neonates. Maternal clinical history included the administration of antenatal steroids. Neonatal clinical history included gestational age at birth, postnatal age at death, birth weight, autopsy weight, kidney weight, neonatal illnesses, medications administered, and cause of death.

Collection of Kidneys at Autopsy and Tissue Sectioning and Staining

Kidneys were excised, weighed, and cut in half in the longitudinal plane. Larger kidneys were further transversely cut. Portions of the kidney were embedded in paraffin blocks, sectioned at 5 μm, and collected onto glass slides. Complete kidney sections with clear evidence of cortex and medulla were selected and stained with hematoxylin and eosin and used for the assessment of nephrogenesis, glomerular size, maturity, and morphology. During all analyses, researchers were blinded to the gestational age and study grouping.

Assessment of Nephrogenesis

Nephrogenic Zone Width.

The width of the nephrogenic zone was measured using image analysis software (Image Pro Plus v. 6.0 for Windows, Media Cybernetics, Silver Spring, Maryland). This method has previously been utilized to assess renal maturity in both human[43] and baboon[16] fetal and neonatal kidneys. Kidney sections were viewed at ×200 magnification and the width of the nephrogenic zone was measured in three to four separate regions. The nephrogenic zone was defined as the area in the outer renal cortex exhibiting developing nephrons in the form of comma and S-shaped bodies. An average nephrogenic zone width was determined for each kidney.

Glomerular Generation Number.

The medullary ray glomerular generation counting method was utilized to estimate the number of glomerular generations formed within the kidney. This method counts the number of glomeruli formed along a medullary ray from the corticomedullary junction to the outer renal cortex, including the glomeruli that form after ureteric branching is complete. This method has been validated by Hinchliffe and colleagues,[44] and also utilized in previous studies to assess renal maturity in preterm human[11–12,45] and baboon neonates.[15,16] In one complete paraffin section from each kidney, approximately five clearly distinguishable medullary rays from separate regions of the kidney section were identified and the number of mature glomeruli along one side of the medullary ray was counted. An average number of glomerular generations per kidney was obtained from the five regions. In circumstances when clear medullary rays were not observed, a straight line was drawn from the corticomedullary junction to the outer renal cortex and all mature glomeruli along the line were counted according to the protocol of Rodriguez and colleagues.[11]

Assessment of Glomerular Size, Maturity, and Morphology

Renal Corpuscle Cross-sectional Area.

Renal corpuscle cross-sectional area was measured using image analysis software (Image Pro Plus v. 6.0 for Windows, Media Cybernetics, Silver Spring, Maryland). A complete section from each kidney was systematically sampled at ×400 magnification with a step length of 1 mm × 1 mm. At each field of view, two glomeruli were chosen for assessment (approximately 200 per kidney). If more than two glomeruli were observed, each glomerulus was assigned a number from 1 to n. With use of a random number table, the first glomerulus (G1) was selected for analysis. The second glomerulus (G2) was selected according to the criteria of Nyengaard and Marcussen,[46] where n is the total number of glomeruli per field of view:

$$G2 = G1 + \frac{n}{2}$$

If the above equation resulted in $G2 > n$, then the following equation was utilized:

$$G1 = G1 - \frac{n}{2}$$

The cross-sectional area of each selected renal corpuscle was then determined by tracing the Bowman's capsule, and the average area

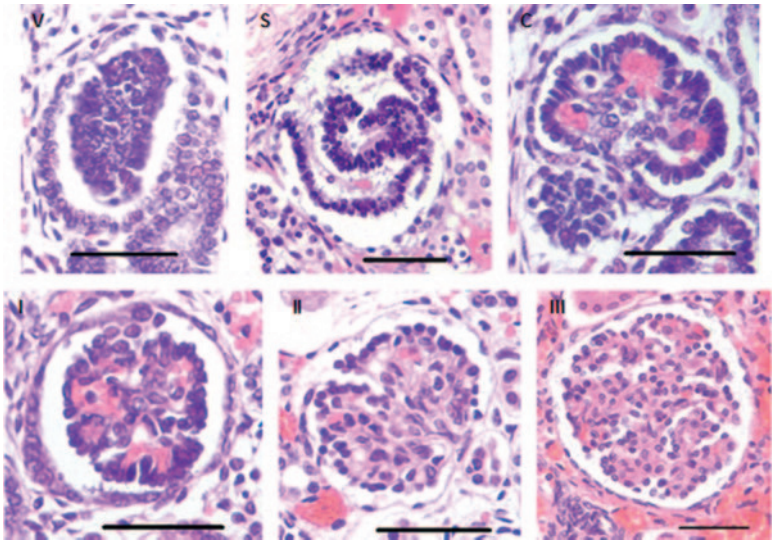

Figure 7. Stages of glomerular maturity in the developing human kidney. The following criteria for the assessment of glomerular maturation are based on Naruse *et al.*[47] and Thony *et al.*[48] **(V) Vesicle:** Condensate of mesenchymal cells formed adjacent to a ureteric branch tip in the outer nephrogenic zone. **(S) Comma-shape and S-shape:** Elongated vesicle develops proximal and distal clefts to form a comma-shaped followed by an S-shaped body. **(C) Capillary loop:** Cells of the lower limb of the S-shaped body differentiate to form an immature, crescent-shaped glomerulus. **(I) Stage I**: Fully formed glomeruli with at least half of the glomerular tuft lined with dark-staining epithelial cells (podocytes). The inner tuft is a dense collection of cells. **(II) Stage II**: Less than half the circumference of the tuft is lined with podocytes, with at least five adjoining. The inner tuft may exhibit some lobulation. **(III) Stage III**: Glomeruli with no podocyte layer surrounding the tuft, and an inner tuft showing lobulation and open capillary loops. There is also evidence of flattening of the parietal epithelial cells lining Bowman's capsule. Bar represents 50 μm.

was calculated for each kidney. Abnormal glomeruli, exhibiting an enlarged Bowman's space and shrunken glomerular tuft, were excluded from the analysis.

Glomerular Maturity and Morphology.
With use of the same sampling method as described above, the maturational stage of all glomeruli present in each field of view was recorded (approximately 300 glomeruli per kidney). The criterion for grading each stage of glomerular maturity is detailed in Figure 7. Immature glomeruli in the early stages of development (stages V, C, and S) were graded according to the criteria of Naruse *et al.*[47] More mature glomeruli (stages I, II, and III) were graded according to the criteria of Thony *et al.*[48] The percentage of abnormal glomeruli at each stage of development per kidney was determined. Glomeruli were classified as abnormal if they exhibited a grossly enlarged Bowman's space and a shrunken glomerular tuft. At each field of view, the numbers of normal and abnormal glomeruli were recorded, and the percentage of abnormal glomeruli per kidney was determined.

Statistical Analysis
All statistical analyses were undertaken using GraphPad Prism v5.03 for windows (GraphPad Software, San Diego). An unpaired *t* test was used to determine statistically significant differences in age and growth characteristics between groups. Linear regression analyses were undertaken to determine correlations between the indices of fetal/neonatal growth and renal morphology (birth weight, kidney weight, nephrogenic zone width, glomerular generation number, renal corpuscle cross-sectional area, glomerular maturity, and the percentage of abnormal glomeruli) *versus* gestational, postnatal, and postconceptional ages. This was followed by an analysis of covariance (ANCOVA) to determine whether there were any significant differences in the slope and *y*-intercept of the linear regression lines between the gestational control, preterm, and preterm + IUGR groups. No differences were found between the preterm group and the preterm + IUGR group in any indices of renal development (kidney weight, kidney weight-to-body weight ratio, nephrogenic zone width, glomerular generation number, renal corpuscle cross-sectional area, and glomerular maturity); therefore, all data were pooled.

A two-way ANOVA was utilized in the assessment of renal development (nephrogenic zone width, glomerular generation number, renal corpuscle-cross-sectional area, and glomerular maturity) with the factors postconceptional age (A), prematurity (P), and the interaction (AxP). This was followed by a Bonferroni *post hoc* test to determine the differences between groups at each age point. Sex differences within groups was also analyzed using a two-way ANOVA and no statistically significant differences were found. The level of significance was accepted at $P < 0.05$.

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DISCLOSURES

None.

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CHAPTER FOUR

GLOMERULAR CAPILLARY LENGTH AND SURFACE AREA IN THE PRETERM LAMB KIDNEY

CHAPTER FOUR DECLARATION

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

Nature of contribution	Extent of contribution (%)
Performed all kidney analyses, all data analysis, wrote the manuscript	70%

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution (%) For student co-authors only
Mar Janna Dahl, Kurt Albertine, M. Jane Black	Involved in study design, performed animal studies, assisted in editing manuscript	30%

Candidate's Signature [redacted] **Date** 30/04/12

Declaration by co-authors

The undersigned hereby certify that:

(31) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(32) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(33) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(34) there are no other authors of the publication according to these criteria;
(35) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(36) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) **Department of Anatomy & Developmental Biology, Monash University**

	Signature	Date
Mar Janna Dahl	[redacted]	24 APR 2012
Kurt Albertine		25/April/2012
M. Jane Black		24/4/12

GLOMERULAR CAPILLARY LENGTH AND SURFACE AREA IN THE PRETERM LAMB KIDNEY

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ABSTRACT

Preterm neonates are born with developmentally immature kidneys, with vascularisation of the immature glomeruli likely ongoing after birth. Preterm birth, and the related increase in blood oxygen concentration, leads to impaired vascular development in a number of diseases of prematurity (including as bronchopulmonary dysplasia and retinopathy of prematurity). Hence, it is likely that glomerular capillary development will also be impaired. The aim of this study was to determine the effects of preterm birth on glomerular capillary length and surface area in a lamb model of preterm birth. Preterm lambs were delivered at 130d gestation (term = 147d), and ventilated after birth (Preterm: n=7). Term lambs were delivered naturally, and breathing was unassisted after birth (Term: n=7). In each group, lambs were euthanised at postnatal day three, and the left kidney from each animal was perfusion-fixed. In epon-araldite embedded sections of inner, mid, and outer renal cortex, stereological techniques were used to calculate average capillary length density and capillary surface area density per renal corpuscle. The results of this study indicated that glomerular size and capillary length vary according to cortical location, with the largest glomeruli localised to the inner renal cortex. Importantly, preterm birth was associated with glomerular capillary dilation; despite significantly reduced renal corpuscle size and capillary length density in the preterm lamb kidney, capillary surface area density was equivalent to term-born controls. In the long-term, if capillary dilation persists it may predispose to the development of glomerulosclerosis, and ultimately result in nephron loss.

INTRODUCTION

Nephrogenesis (the development of nephrons in the kidney) is normally ongoing in late gestation. Even after final nephron endowment is attained (by 32-36 weeks gestation (19, 43)) the vascularisation and maturation of glomeruli may be still ongoing (46). As such, preterm neonates (delivered prior to 37 completed weeks of gestation) are born with a developmentally immature kidney (17). Impaired vascularisation is known to be an underlying factor in a number of morbidities of prematurity such as bronchopulmonary dysplasia and retinopathy of prematurity (41, 48); therefore, it is likely that the development of the glomerular capillaries will also be affected. In this regard, morphologically abnormal glomeruli with shrunken glomerular tufts are commonly observed in the outer renal cortex of the kidney in human neonates born preterm (38, 43), and also in animals models of preterm birth (18, 44, 45), suggestive of impaired glomerular development or injury.

Impaired vascularisation in other organ systems following preterm birth is related to high oxygen concentrations in the extrauterine environment (40). The intrauterine environment is relatively hypoxic (13, 37), which is optimal for renal organogenesis (49). Preterm neonates at the time of birth are abruptly exposed to high oxygen concentrations relative to the intrauterine environment (21% to 100% O_2 is used during resuscitation and ongoing supplemental oxygen therapy (33, 47)), with arterial blood oxygen saturation rapidly increasing after birth (21, 34). These high oxygen concentrations are known to downregulate the expression of vascular endothelial growth factor (VEGF) (15, 25), which is essential for glomerular capillary growth and development (11). In addition, there is a significant increase in cardiac output and renal blood flow after birth (1, 30). It is conceivable that the resulting high pressures within the immature capillary may lead to vascular injury, as has been evidenced previously in the aortic arch of a sheep model of preterm birth (3).

It is currently unknown, however, what effect preterm birth has on the immature vasculature of the glomeruli. Using stereological techniques, the aim of this study was to assess glomerular capillary length and surface area following moderate preterm birth at 130 days gestation (term = 147 days) in a lamb model. In the lamb, nephrogenesis is

completed prior to delivery at approximately 120 days gestation (16), however the vascularisation of immature glomeruli is likely to be still ongoing at 130 days gestation (46).

METHODS

Animals

Preterm lambs were delivered via caesarean at 130 days gestation (term = 147 days), and were ventilated after birth (Preterm: n=7). Control lambs were delivered naturally at term, and their breathing was unassisted after birth (Control: n=7). All animals were euthanised at postnatal day three (approximately 72 hours after birth). For a detailed description of the animal care procedures, see Reyburn *et al*. (35). All protocols adhered to the American Physiological Society/National Institute of Health guidelines for the humane use of animals for research, and were approved by the Institutional Animal Care and Use Committee at the University of Utah Health Sciences Center.

Tissue processing

At necropsy, the left kidney of each lamb was excised and perfusion-fixed via the renal artery using 10% buffered formalin. Kidneys were cleaned of connective tissue and fat, decapsulated, cut into quarters, and weighed. Two opposing quarters were subsequently selected and sliced using a razor blade device into slices of 2 mm thickness. From these, every third slice was selected (beginning from a random starting point). In each selected slice, at a position selected at random, an approximately 2 mm wide strip of tissue, extending from the outer cortex to the medulla, was cut. Each strip of tissue was then cut equally into three cubes of tissue representing the inner, mid and outer renal cortex. Four cubes of cortex per each of the three regions (12 per kidney) were post-fixed in osmium tetroxide before being embedded in epon araldite (5). Epon araldite blocks were sectioned at 1 µm (with approximately ten sections collected per slide) and stained with toluidine blue.

Sampling and photography of glomeruli

Two glomeruli per section (24 per kidney) were chosen for analysis. In order to randomly sample the glomeruli in an unbiased manner, one complete tissue section per slide was viewed at low magnification, and each glomerulus present in the section was numbered from 1 to *n*. Incomplete glomeruli located at the edges of the section were excluded. Using a random number table, the first glomerulus (G1) was selected for analysis. The

second glomerulus (G2) was selected according to the criteria of Nyengaard and Marcussen (28) where *n* is the total number of glomeruli per field of view:

$$G2 = G1 + \frac{n}{2}$$

If the above equation resulted in G2 > *n*, then the following equation was utilised:

$$G2 = G1 - \frac{n}{2}$$

Each selected glomerulus was then photographed using a 100X oil-immersion lens (Image Pro Plus, version 6.0). Viewed at a total magnification of 1700X, imaging software (Adobe Photoshop CS5 Extended, version 12.0.4) was used to trace the inner margin of the Bowman's capsule, and to trace the boundary of the capillary loops within the glomerular tuft (Figure 1).

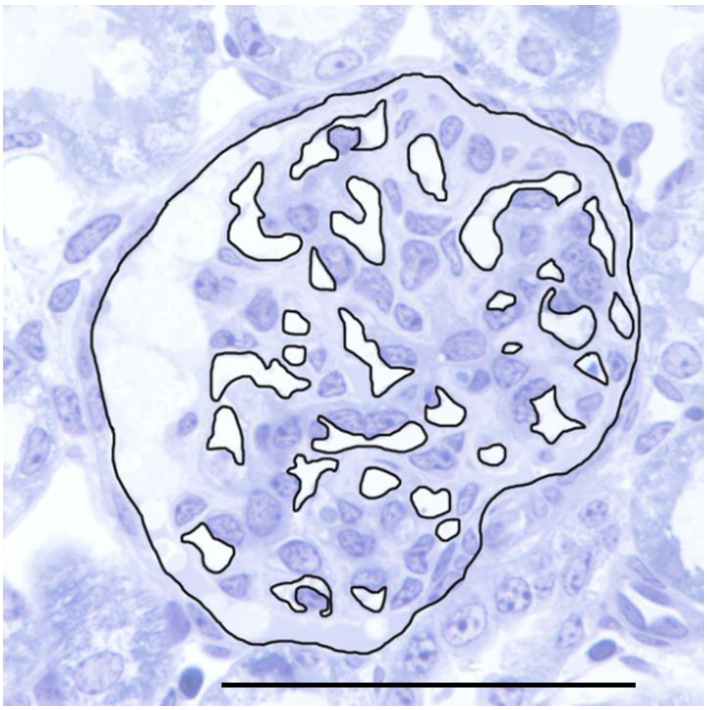

Figure 1: Representative photomicrograph of a renal corpuscle (preterm lamb, mid-cortical region) with tracings along the inner boundary of the Bowman's capsule and capillary walls. These tracings are required for the calculation of renal corpuscle size, and capillary length and surface area. Bar = 50 µm.

Assessment of renal corpuscle size, capillary length density, and surface area density

Renal corpuscle cross-sectional area and capillary length and surface area density were determined using previously described stereological techniques (4, 5). A 15 mm x 15 mm unbiased orthogonal grid was superimposed over the glomerular tracings. The number of grid points overlaying the renal corpuscle (P_{corp}), the number of capillary profiles within the glomerulus (Q^-), and the number of capillary boundaries that intersect with the horizontal and vertical lines of the superimposed grid (I_{cap}) were recorded.

The following calculation was utilised to calculate renal corpuscle cross-sectional area:

*Renal corpuscle cross-sectional area (mm^2) = P_{corp} * a(p)*

Where P_{corp} is the number of grid points overlaying the renal corpuscle, and a(p) is the area associated with each grid point.

Capillary length density per renal corpuscle was calculated by:

*Lvcap, corp (mm/mm^3) = (2 * Q^-) / (Pcorp * a(p))*

Where 2 is a constant that accounts for the capillaries being isotropic, Q^- is the number of capillary profiles, Pcorp is the number of grid points overlaying the renal corpuscle, a(p) the area associated with each grid point.

Capillary surface area density per renal corpuscle was calculated by:

*Svcap, corp (mm^2/mm^3) = (2 * I_{cap}) / (Pcorp * k * d)*

Where 2 is a constant that accounts for the capillaries being isotropic, I_{cap} is the number of intersections of horizontal and vertical gridlines with capillary boundaries, P_{corp} is the number of grid points overlaying the renal corpuscle, k is a constant accounting for the number of lines associated with a grid point (k=2 for an orthogonal grid), and d is the distance between gridlines divided by the total magnification.

Calculations for individual glomeruli were averaged separately for each of the inner, mid and outer cortical regions per kidney.

Assessment of renal morphology

In each kidney, the presence or absence of morphologically abnormal glomeruli (exhibiting an enlarged Bowman's space and shrunken glomerular tuft) was recorded. To do this, an examination of all sections collected per kidney (approximately 10) was conducted at a low magnification.

Statistical analysis

Data were analysed using GraphPad Prism (version 5.03 for Windows; GraphPad Software, CA, USA), with all data presented as the mean ± the standard error of the mean (SEM). Kidney weights were compared between preterm and control groups using a two-tailed unpaired Student's T-test. Renal corpuscle cross-sectional area, capillary length density and capillary surface area density were analysed using a two-way analysis of variance (ANOVA), with the factors preterm birth (p_P), region of cortex (p_R), and their interaction (p_{PxR}). Statistical significance was accepted at the level of $p<0.05$.

RESULTS

Kidney weight

Left kidney weight averaged 15.7 ± 1.3 g in the control group, and 9.6 ± 0.8 g in the preterm group; kidney weight was significantly less in the preterm animals than those born at term (Figure 2).

Renal corpuscle size

Renal corpuscle cross-sectional area was significantly affected by the localisation of the glomerulus within the renal cortex, as well as preterm birth (Figure 3). Renal corpuscles located in the inner renal cortex were largest, whereas those in the outer renal cortex were significantly smaller. Renal corpuscle size was also significantly smaller in the preterm animals (mean: 4.25 ± 0.17 x 10^{-3} mm^2) compared to those born at term (mean: 5.43 ± 0.20 x 10^{-3} mm^2).

Capillary length and surface area density

Average capillary length density per renal corpuscle (Figure 4A) was significantly affected by the localisation of the renal corpuscle within the renal cortex; capillary length density was greatest in glomeruli within the inner cortex and significantly less in the outer cortical glomeruli. Overall, capillary length density was significantly reduced in the preterm kidneys compared to the term controls. There was no effect of either the cortical location or preterm birth on average capillary surface area density per renal corpuscle (Figure 4B).

Renal morphology

Overall, there was no overt difference in renal cortical morphology between the preterm and control animals, and there was no evidence of morphological glomerular abnormalities in the kidneys of the preterm lambs.

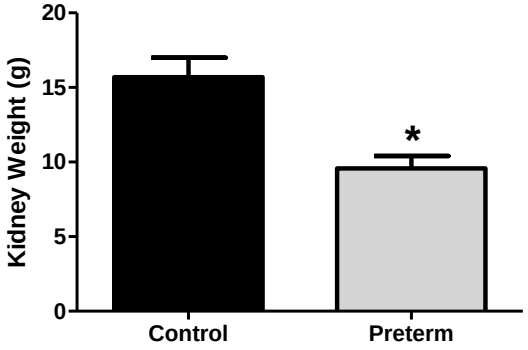

Figure 2: Left kidney weight in term control animals (black) compared to preterm animals (grey). Kidney weight was significantly greater in the term controls *p=0.002.

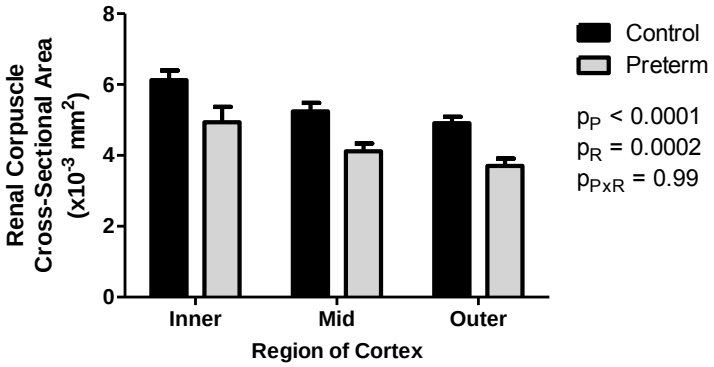

Figure 3: Renal corpuscle cross-sectional area in control (black) and preterm (grey) groups. There was a significant effect of both preterm birth (p_P<0.04) and region of cortex (p_R<0.03) on renal corpuscle size.

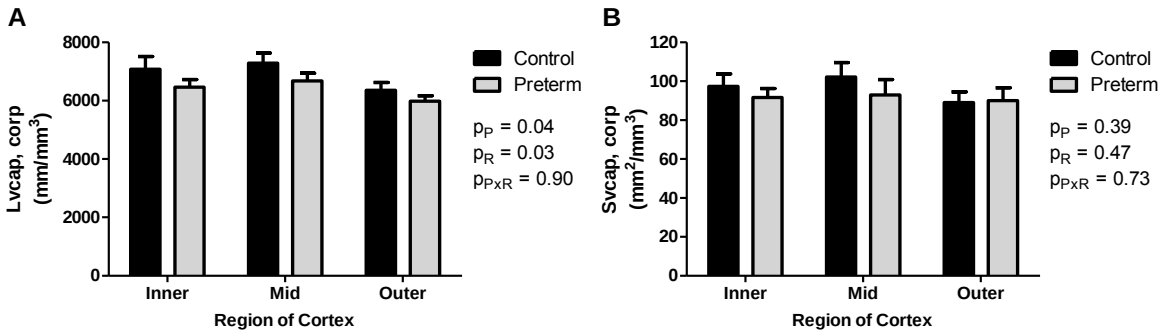

Figure 4: Average capillary length density per renal corpuscle (**A**) and average capillary surface area density per renal corpuscle (**B**) in control (black) and preterm (grey) groups. There was a significant effect of both preterm birth (p_P<0.04) and region of cortex (p_R<0.03) on capillary length density (A).

DISCUSSION

Through this assessment of glomerular capillary growth in the moderately preterm lamb kidney, we have shown that glomerular size and capillary length vary according to cortical location. Importantly, preterm birth was associated with glomerular capillary dilation; despite significantly reduced renal corpuscle size and capillary length density in the preterm lamb kidney, capillary surface area density was equivalent to term-born controls.

Renal corpuscle size and glomerular capillary length vary according to cortical region

In both the moderately preterm lamb kidney and the term lamb kidney at postnatal day three, renal corpuscle size significantly differed according to the localisation of the glomeruli within the renal cortex. Glomeruli present in the outer renal cortex were significantly smaller than glomeruli located within the mid and the inner renal cortex (where glomeruli were observed to be the largest). Accordingly, there was also an effect of cortical region on capillary length density, with the smaller glomeruli in the outer renal cortex exhibiting a reduced capillary length density than the larger inner-cortical glomeruli.

The distribution pattern of glomerular size observed in the current study has previously been noted in human kidneys during gestation and into childhood (27, 42). In contrast, however, studies in adults have demonstrated that although significant heterogeneity exists in glomerular size within individual kidneys, regional differences are not observed (32, 39, 51). The difference in glomerular size and capillary length according to cortical region likely relates to the maturity of the glomeruli; by the completion of nephrogenesis, the oldest nephron is positioned near to the medulla, whereas the most recently formed nephrons (the smallest) are positioned in the outer renal cortex beneath the renal capsule (29). It is therefore probable that the development and vascularisation of juxtamedullary glomeruli, at any given time point, is at a more advanced stage than in those glomeruli that were most recently formed. Furthermore, the anatomy and functionality of glomeruli is known to differ according to cortical region. For example, glomeruli localised to the inner cortex, with a long loop of Henle extending into the medulla, are noticeably different in comparison to short-looped glomeruli originating within the mid and outer renal cortex (2, 39). It is to be noted, however, that capillary

surface area density per renal corpuscle (measure of filtration surface area) was not different between glomeruli in different cortical regions, suggesting equivalent capacities for glomerular filtration regardless of the location of the glomerulus within the renal cortex.

Preterm birth leads to the dilation of glomerular capillaries

Despite the significantly reduced renal corpuscle size and capillary length density evident in the glomeruli of the preterm animals, capillary surface area density was equivalent to the term controls. This suggests that substantial dilation of the shorter glomerular capillaries occurs in the preterm kidney after birth. A comparison with results derived from a previous study, conducted in gestational control lambs, demonstrates that renal corpuscle size and capillary length density are very similar in the preterm lamb (delivered at 130 days gestation and examined at postnatal day 3) to the levels expected *in utero* (26). Average capillary surface area density per renal corpuscle, however, was approximately ten-fold larger in the preterm kidneys compared to the gestational controls (26); this result further supports the finding of glomerular capillary dilatation after birth.

The increased dilation of glomerular capillaries observed in the preterm kidney is likely due to the increased functional demands on the kidney and/or the haemodynamic changes that occur after birth; namely, increases in renal blood flow (1, 30) and blood oxygen saturation (21, 34). Indeed, it has been shown that raised intraglomerular blood pressure results in mechanical stretch of the capillary wall (23), which has an anti-proliferative effect on podocytes (31), and a pro-proliferative effect on mesangial and vascular smooth muscle cells (7, 23, 36). If stabilisation of the capillary through the action of the glomerular basement membrane and podocytes fails, perhaps due to structural immaturity of the glomerulus, the increased pressure within the lumen will result in dilation of the capillary (22, 23). Additionally, increased blood oxygen saturation levels are known to downregulate the expression of VEGF (15, 25) which ultimately results in the structural breakdown of the glomerular filtration barrier (12, 50). Besides damaging the structural integrity of existing glomerular capillaries, it is hypothesised that

attenuations in VEGF expression would also impair any ongoing vascularisation in this model (11).

In previous studies of human and baboon preterm neonates, we have reported the presence of morphologically abnormal glomeruli, with shrunken glomerular tufts, in the outer renal cortex of the kidney (18, 43-45). The abnormal glomeruli were only observed in the outer renal cortex, suggesting that it is only glomeruli that were newly formed that are at risk of impairment (17). Interestingly, however, no abnormal glomeruli were observed in the preterm lamb kidneys in the current study. The difference between this study and those conducted previously is that nephrogenesis was completed in the lamb prior to the induction of preterm birth. Therefore, the absence of abnormal glomeruli in this model supports the premise that the glomerular abnormalities are the result of impaired nephrogenesis, and/or injury, to the developing glomeruli.

Potential outcomes of capillary dilation

In this study, it was not possible to determine whether the increased capillary dilation in the preterm kidney represents a pathological process that will lead to subsequent renal injury. Based on Laplace's law (the tension within a sphere is a product of the pressure within the sphere and its radius (24)), however, it has been suggested that enlarged capillary lumen size would result in an increased susceptibility to hypertensive damage (10, 14); this is important given that there is strong epidemiological evidence linking preterm birth to the development of hypertension in children and adults (6, 8). Capillary dilation also increases the risk of adhesion of the glomerular tuft to the Bowman's capsule, thus leading to the development of focal segmental glomerulosclerosis (FSGS) and subsequent nephron loss (9, 23). In this regard, there has been one study to describe renal pathology in preterm-born individuals in late childhood and adulthood; in a group of six people born at 22-30 weeks gestation, and assessed at 15-52 years of age, there were typical findings of post-adaptive focal segmental glomerulosclerosis in the absence of other risk factors for FSGS (20). Given that glomerular hypertrophy is already evident in the preterm lambs at the beginning of life, in future studies it would be beneficial to assess the kidneys of preterm-born lambs at later time points in order to determine

whether glomerulosclerosis and/or impaired vascular development (reductions in capillary length) does occur in this model as we had originally hypothesised.

Conclusions: The findings from this study demonstrate that as a result of moderate preterm birth in the lamb, glomerular capillary dilation occurs in glomeruli located throughout the renal cortex. If the glomerular capillary dilation persists, this may predispose to glomerulosclerosis, nephron loss, and thereby have long-term deleterious effects on renal function in individuals born preterm.

GRANTS

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CHAPTER FIVE

NEONATAL HYPEROXIA EXPOSURE LEADS TO GLOMERULAR HYPERTROPHY IN THE ADULT MOUSE KIDNEY

CHAPTER FIVE DECLARATION

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

Nature of contribution	Extent of contribution (%)
Performed majority of kidney analyses, performed all data analysis, and wrote the manuscript	60%

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution (%) For student co-authors only
Megan O'Reilly	Performed animal studies	15%
Kimberley Ong	Performed some kidney analyses	5%
Richard Harding, Foula Sozo, M. Jane Black	Involved in the design of experiments, performed animal studies, and assisted with editing the manuscript	20%

Candidate's Signature [redacted] **Date** 30/04/12

Declaration by co-authors

The undersigned hereby certify that:

(37) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.

(38) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(39) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(40) there are no other authors of the publication according to these criteria;

(41) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(42) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) **Department of Anatomy & Developmental Biology, Monash University**

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NEONATAL HYPEROXIA LEADS TO GLOMERULAR HYPERTROPHY IN THE ADULT MOUSE KIDNEY

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ABSTRACT

Preterm neonates are born while nephrogenesis is ongoing and are commonly exposed to factors in the extrauterine environment that may impair renal development. Supplemental oxygen therapy exposes the preterm infant to a hyperoxic environment which may induce oxidative stress. Our aim was to determine the immediate and long-term effects of exposure to hyperoxia, during the period of postnatal nephrogenesis, on renal development. Newborn mice (C57BL/6) were kept in a normoxic (room air, 21% oxygen) or a controlled hyperoxic (65% oxygen) environment from birth to postnatal day 7 (P7). From P7, animals were maintained in room air until adulthood at postnatal day 56 (P56). Pups were assessed for glomerular maturity and renal corpuscle cross-sectional area at P7 (short-term survival group: control n=14; hyperoxic n=14). Nephron number and renal corpuscle size were determined stereologically at P56 (long-term survival group: control n=14; hyperoxic n=14). At P7, there was no effect of hyperoxia on glomerular size or maturity. In adulthood (P56), kidney weight relative to body weight, and kidney volume were not different between hyperoxia-exposed animals and controls. At P56, nephron number averaged 10,140 ± 433 in the controls and 9,425 ± 327 in the hyperoxia-exposed animals, with no significant difference between treatment groups. However, in adult mice renal corpuscle volume was significantly greater in the hyperoxia-exposed animals compared to the controls ($p=0.03$). As glomerular hypertrophy is a key antecedent to renal disease, our findings have important implications for the long-term renal health of preterm infants exposed to hyperoxic gas during the neonatal period.

INTRODUCTION

Nephrogenesis (the formation of nephrons in the kidney) is usually complete by about 32-36 weeks of human gestation (12, 33). Hence, preterm infants are often born at a time when nephrogenesis is ongoing; in these infants, nephrogenesis continues after birth (9, 33). It is apparent that renal development may be impaired after preterm birth, with studies demonstrating accelerated postnatal renal maturation (33) and reduced formation of glomeruli (30). Of particular concern, glomerular abnormalities are often present in the outer renal cortex of preterm infants, where the most recently formed glomeruli are located (9, 30, 33, 34). In preterm infants, the number of morphologically abnormal glomeruli within the kidneys is variable, with a high proportion of glomerular abnormalities in some kidneys, whereas other kidneys appear to be unaffected (33). Although the cause of the glomerular abnormalities is unknown, the abnormal glomeruli are located only within the outer renal cortex, and are in an immature stage of development (33), suggesting that it is the nephrons that are formed in the extrauterine environment that are 'at risk.' Therefore, it may be factors associated with the postnatal care of the infant that are leading to the abnormalities.

Supplemental oxygen therapy is common practice in the care of preterm neonates, and high levels of oxygen in the neonatal period may adversely impact upon nephrogenesis. It has been shown in explants of rat kidneys that low oxygen concentrations (1-3%) are optimal for renal vascular and tubular development (35). The intrauterine environment is relatively hypoxic (6, 29), with arterial oxygen saturation rising immediately after birth (18, 28). Preterm neonates at the time of birth are therefore abruptly and prematurely exposed to high oxygen concentrations relative to the intrauterine environment. It is now recognised that even brief exposure to high oxygen concentrations can lead to oxidative stress (37, 38); free radicals are reported to cause cellular injury and cell death when the antioxidant capacity of the neonate is overwhelmed (21). Preterm infants are particularly vulnerable as they have low concentrations of antioxidants (8, 19). In this regard, common diseases of prematurity such as bronchopulmonary dysplasia, retinopathy of prematurity, necrotising enterocolitis, patent ductus arteriosus and periventricular leukomalacia are all strongly linked to oxidative stress (31).

In the kidney, oxidative stress is associated with proximal tubule injury in human neonates (26, 40). It is likely that it may also lead to injury to the glomerular capillaries and thus may be the cause of the glomerular abnormalities in kidneys of preterm infants. Early life exposure to hyperoxia in rats (80% oxygen from postnatal day 3-10, a time when nephrogenesis is still ongoing) has been shown to lead to hypertension, microvascular rarefaction, vascular dysfunction, and a 25% reduction in nephron endowment in adulthood (41), strongly suggesting that hyperoxia during nephrogenesis adversely impacts on renal development.

Hence, the aim of the present study was to determine whether exposure to hyperoxia during postnatal nephrogenesis in the neonatal mouse leads to abnormal glomerular formation, size, and maturation. In mice, nephrogenesis begins during mid-gestation and continues until approximately 7 days after birth (3, 11). After exposing pups to moderately high oxygen concentrations (65% O_2) during the neonatal period, we examined the kidneys at two time points: immediately following hyperoxic exposure at postnatal day 7 (P7) and in early adulthood at P56. At P7, we assessed the immediate consequences of neonatal hyperoxia on glomerular morphology, size, and maturation. At P56, nephron number was stereologically measured, which indicates the final complement of nephrons formed. The size of the glomeruli at P56 was also determined.

METHODS

Animals

Mice (C57BL/6) were born naturally at term and exposed to hyperoxic gas (hyperoxia group, 65% O_2), or room air (control group, 21% O_2), from the time of birth (P0) until P7. Pregnant dams in the hyperoxia group were exposed to 65% O_2 from 0.5 days prior to birth such that pups were born into the hyperoxic environment. O_2 and CO_2 concentrations in the mouse housing chambers were continuously monitored (Servoflex MiniMP 5200; Servomex) for the duration of the hyperoxia exposure period; CO_2 concentrations did not rise throughout the exposure period. We used 65% O_2 to avoid maternal and neonatal death through severe oxygen toxicity; this is a lower O_2 concentration than has been used in other recent studies (36, 41). All studies were approved by the Monash University Animal Ethics Committee and the treatment and care of animals conformed to the National Health and Medical Research Council of Australia's *Code of Practice for the Care and Use of Animals for Scientific Purposes*.

Experimental Groups

Fourteen control litters (7 short-term survival and 7 long-term survival) and 14 hyperoxia-exposed litters (7 short-term survival and 7 long-term survival) were analysed in this study. In all groups one male and one female pup, selected at random, were analysed per litter.

Short-term survival (P7) group: Hyperoxia-exposed pups were continuously exposed to hyperoxic gas (65% oxygen) from P0 to P7 (Hyperoxia; male n=7, female n=7). Controls breathed room air (21% oxygen) from P0 to P7 (Control; male n=7, female n=7). At P7, the offspring underwent necropsy for collection of the kidneys. Pups were weighed at birth and at P7.

Long-term survival (P56) group: Hyperoxia-exposed pups were continuously exposed to hyperoxic gas (65% oxygen) from P0 to P7, and then raised in room air (21% oxygen) until adulthood at P56 (Hyperoxia; male n=7, female n=7). Controls were exposed to room air (21% oxygen) from P0 to P56 (Control; male n=7, female n=7). Offspring were weaned at

P21, and were weighed weekly from birth to P56. At P56, the mice underwent necropsy for collection of the kidneys.

Tissue Processing

All kidneys collected at necropsy were cleaned of connective tissue and immersion-fixed in 10% buffered formalin. The entire right kidney from each animal in the short-term survival groups was paraffin-embedded and sectioned at 7 µm. Every 10^{th} section was collected and stained with haematoxylin and eosin. The right kidney from each animal in the long-term survival groups was weighed, cut in half along the coronal axis, and embedded in glycolmethacrylate. Glycolmethacrylate blocks (one per kidney) were serially sectioned at 20µm, with every 10^{th} and 11^{th} section collected and stained with haematoxylin and eosin. During all analyses, researchers were blinded as to the sex and experimental grouping of each animal.

Short-Term Survival Group: Renal Corpuscle Cross-Sectional Area and Glomerular Maturity

Haematoxylin and eosin stained paraffin sections of kidneys from the short-term survival groups (P7) were used in the assessment of the cross-sectional area and maturity of renal corpuscles; the renal corpuscle comprises the Bowman's capsule, Bowman's space and glomerular tuft. Every 10^{th} section from each kidney was systematically sampled at 1500x magnification, at a step length of 1mm x 1mm in the x- and y-axis. At each field of view, two glomeruli were randomly chosen for assessment, as described previously (33). The cross-sectional area of each renal corpuscle was determined by tracing the perimeter of the Bowman's capsule using image analysis software (Image Pro Plus, v6.0 for Windows; Media Cybernetics, MD, USA); the average renal corpuscle cross-sectional area was then calculated per kidney (33).

For each field of view, using the uniform systematic sampling method described above, the stage of maturation of all glomeruli within each sampled field was recorded (33). Glomeruli were classified as stage 0 if they were still developing (at the vesicle, comma-shape, S-shape or capillary loop stages of development). Stage 1 comprised immature fully formed glomeruli with at least half of the glomerular tuft lined with dark-staining

epithelial cells, and a densely cellular glomerular tuft. Stage 2 glomeruli presented less than half of the glomerular circumference lined with darkly-stained cells, and Stage 3 glomeruli (most mature) had no dark-staining layer of cells surrounding the glomerular tuft, and a more open glomerular tuft (33).

Long-Term Survival Group: Kidney Volume, Renal Corpuscle Volume, and Nephron Number

Haematoxylin and eosin stained glycolmethacrylate sections of kidneys from the long-term survival group (P56) were used in the stereological estimation of kidney volume, nephron number and renal corpuscle volume. Kidney volume was estimated using the Cavalieri principle (10, 32). Three pairs of intact 10^{th} and 11^{th} sections from each kidney (each section showing two faces of kidney tissue, as the kidneys were cut in half prior to embedding) were used in the estimation of nephron number. The number of glomeruli (and thereby nephrons) was estimated using an unbiased physical disector/fractionator technique (1, 25, 32). Renal corpuscle volume was also stereologically measured (1, 25, 32); glomerular tuft volume was not used as a measure of glomerular size due to the likely collapse of capillaries following tissue processing procedures (1). These techniques have been previously described in detail (32).

Statistical Analysis

Data were analysed using GraphPad Prism (v5.03 for Windows; GraphPad Software, CA, USA), with data presented as the mean ± the standard error of the mean (SEM). Data at each age point (P7 and P56) were analysed using a two-way analysis of variance (ANOVA), with the factors sex (p_S), hyperoxic gas exposure (p_H), and their interaction (p_{SxH}). To identify differences between individual groups, a Bonferroni post-hoc test was conducted following the two-way ANOVA. The growth trajectory of animals from birth to P56 was analysed using a two-way ANOVA with repeated measures, with the factors postnatal age (p_A), hyperoxic gas exposure (p_H), and their interaction (p_{SxH}); this was also followed by a Bonferroni post-hoc test. Body weights at the two endpoints, P7 and P56, were further assessed using an unpaired two-tailed Student's t-test. At P56, linear regression analyses were also undertaken to determine correlations between kidney volume and body weight, and between nephron number and kidney weight and volume. An analysis of co-

variance was subsequently applied to determine whether there were any significant differences in the slope and y-intercept of the linear regression lines between the control and hyperoxia-exposed groups. Statistical significance was accepted at the level of $p \leq 0.05$.

RESULTS

Short-Term Survival Group

Body weight: There was no significant difference between experimental groups, or between sexes, in birth weights. Body weights at P7 were significantly lower in the hyperoxia-exposed animals compared to controls ($p=0.05$), with no effect of sex on body weight (Figure 1A).

Renal corpuscle cross-sectional area: The cross-sectional area of the renal corpuscle at P7 averaged 1561 μm^2. There was no significant difference in renal corpuscle cross-sectional area between sexes, or between hyperoxia-exposed animals and the controls (Figure 1B).

Renal morphology: There was no observable difference in renal cortical morphology between control and hyperoxia-exposed animals (Figure 2), and there was no evidence of morphological glomerular abnormalities in the kidneys of hyperoxia-exposed mice. There was evidence of ongoing nephrogenesis in all kidneys examined at P7, with glomeruli in stage 0 of development (most immature stage) present in the outer renal cortex of all control and hyperoxia-exposed animals (Figure 2).

Glomerular maturity: The percentage of glomeruli at each maturational stage is shown in Figure 3. There was no significant effect of sex or hyperoxia-exposure on glomerular maturity. In all kidneys, the majority of glomeruli (approximately 64% per kidney) were at stage 1 of development (immature), 19% were at stage 2 (intermediate), and an average of 8% were at both stage 0 (developing) and stage 3 (mature).

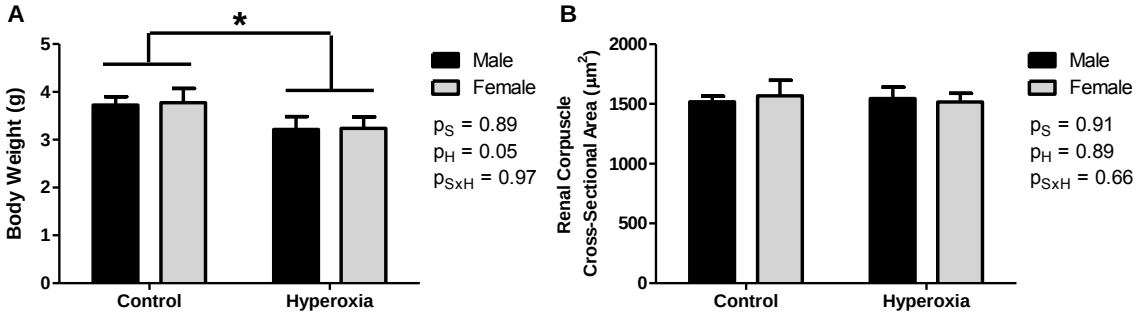

Figure 1: Body weight (**A**) and renal corpuscle cross-sectional area (**B**) of control and hyperoxia-exposed animals at P7 (male, black; female, grey). Hyperoxia exposure (p_H) resulted in significantly reduced body weight compared to controls. There was no effect of sex (p_S) on body weight at P7 (A). There was no effect of sex (P_S) or hyperoxia-exposure (p_H) on renal corpuscle cross-sectional area (B). *$p \leq 0.05$ control versus hyperoxia.

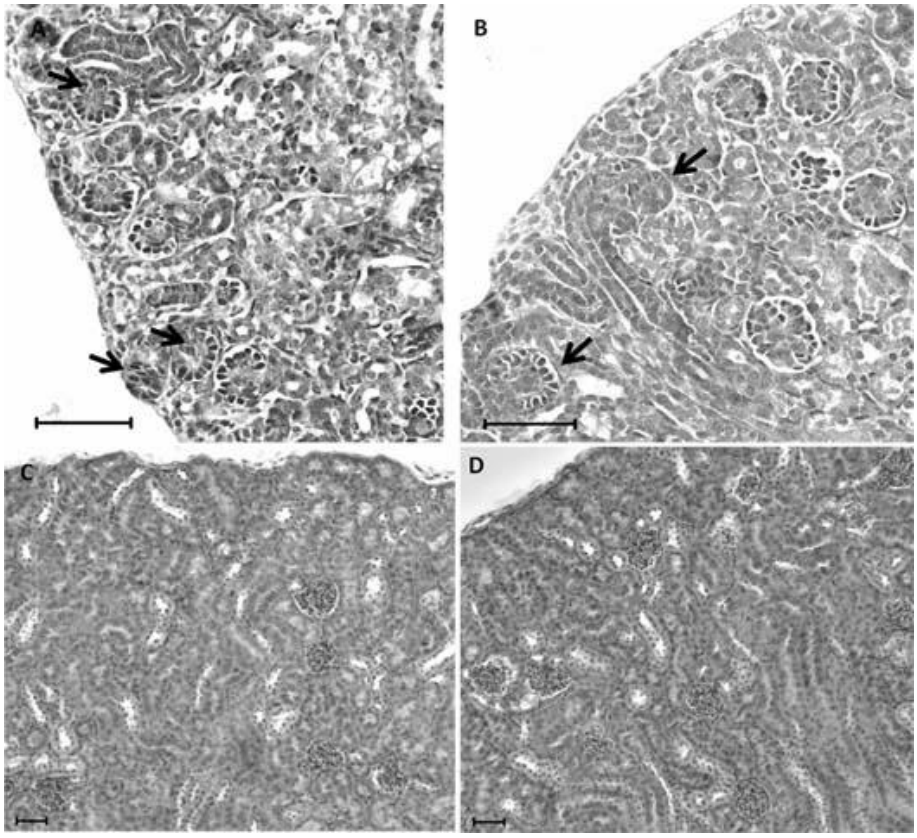

Figure 2: Representative photomicrographs of renal cortical morphology in neonatal (P7) and adult (P56) mice. Developing glomeruli (stage 0) were present in the outer renal cortex of all kidneys examined in the control (**A**) and hyperoxia-exposed (**B**) groups at P7 (arrows). In adult kidneys at P56, there was no discernible difference in renal morphology between control (**C**) and hyperoxia-exposed (**D**) animals. There was no evidence of morphologically abnormal glomeruli in the outer renal cortex of hyperoxia-exposed kidneys. Scale bar represents 50μm.

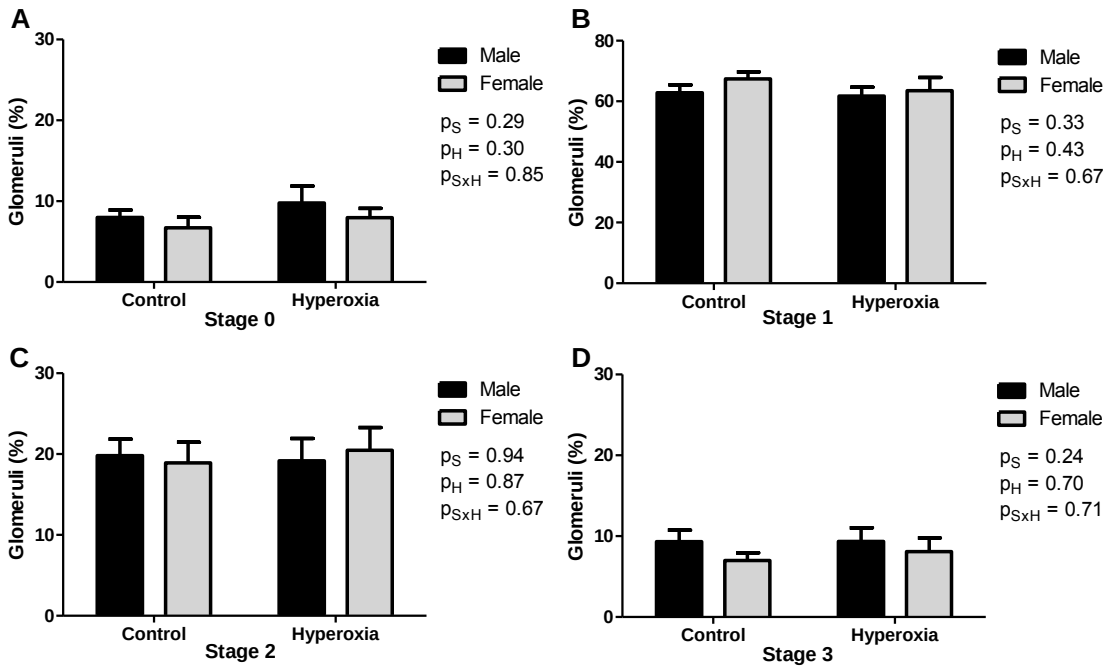

Figure 3: Percentage of glomeruli at each stage of maturity: Stage 0 (**A**; developing glomerulus), stage 1 (**B**; immature glomerulus), stage 2 (**C**; intermediate glomerulus), and stage 3 (**D**; mature glomerulus). Males are shown in the black bars and females in the grey bars. There was no effect of sex (p_S) or hyperoxia-exposure (p_H) on the percentage of glomeruli at any stage of glomerular maturity.

Long-Term Survival Group

Body weight: Mean body weight increased from 1.3 ± 0.0 g at birth, to 25.0 ± 0.4 g in males and 19.1 ± 0.3 g in females by P56. The postnatal increase in body weights of females followed the same trajectory as males until P28; from P28 to P56, males were significantly heavier than females ($p<0.001$). There was no overall effect of hyperoxia on the growth trajectory of male and female animals between birth to P56 (Figure 4); however, body weights were significantly reduced in both male and female hyperoxia-exposed animals at P7.

Kidney weight and volume: At P56, absolute right kidney weights (Figure 5A) and volumes (Figure 5C) were significantly smaller in females compared to males, with no effect of hyperoxia on kidney size. However, a strong trend ($p=0.06$) was evident for an increased kidney weight relative to body weight in females compared to males (Figure 5B). In both the control and hyperoxia-exposed groups there was a significant positive linear correlation between kidney weight and body weight (Control: $r^2= 0.84$, $p=0.0002$; Hyperoxia: $r^2=0.67$, $p=0.004$), as well as kidney volume and body weight (Control: $r^2= 0.72$, $p<0.0001$; Hyperoxia: $r^2=0.56$, $p=0.002$) at P56.

Renal morphology: There was no discernible difference in renal cortical morphology between control and hyperoxia-exposed animals at P56, with no evident morphological glomerular abnormalities (Figure 2).

Nephron number and density: At P56, nephron number (Figure 6A) ranged from 8,191 to 12,860 in the control animals (mean: 10,140 ± 433), and from 7,212 to 10,980 in the hyperoxia-exposed animals (mean: 9,425 ± 327); there was no significant effect of sex or early life hyperoxia exposure on nephron number. Nephron density (number of nephrons per mm^3 of kidney tissue) was significantly greater in females compared to males (Figure 6B). There was no significant correlation between nephron number and kidney weight, kidney volume, or body weight.

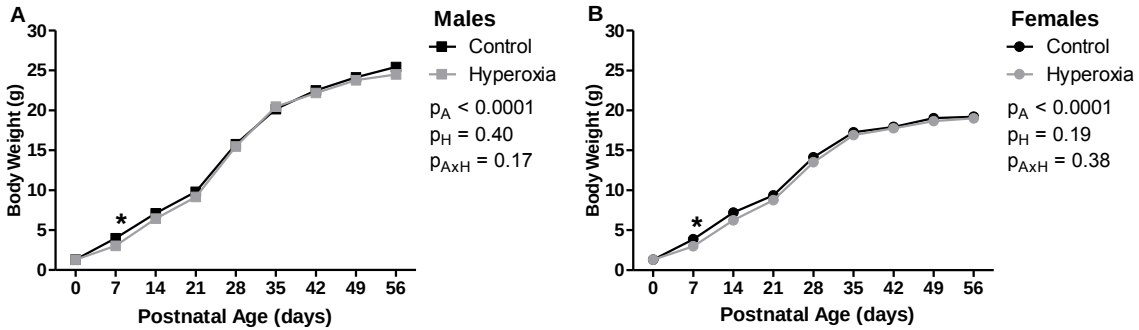

Figure 4: Growth trajectory of male (**A**) and female (**B**) animals from birth to P56. Controls are shown by black lines and hyperoxia-exposed by grey lines. Body weights significantly increased with increasing postnatal age (p_A). Hyperoxia-exposure significantly reduced body weight at P7 in both male and female animals, but there was overall no effect of hyperoxia exposure on the growth trajectory from birth to P56 (p_H). *p<0.01 control versus hyperoxia.

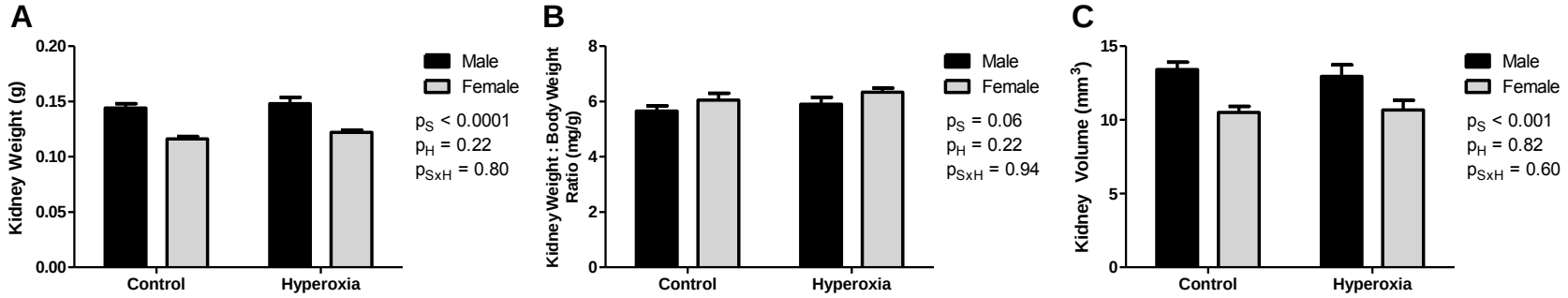

Figure 5: Kidney weight (**A**), kidney weight to body weight ratio (**B**) and kidney volume (**C**) in males (black) and females (grey) in control and hyperoxia-exposed groups. There was a significant effect of sex (p_S) on kidney weight (A) and kidney volume (C), with a greater kidney size evident in the males. There was no effect of hyperoxia-exposure (p_H) on any parameter of kidney size.

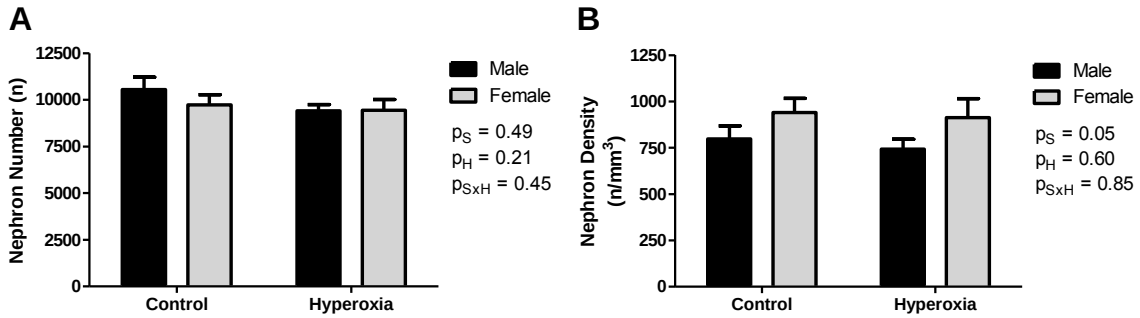

Figure 6: Nephron number (**A**) and nephron density (**B**) in males (black) and females (grey) in control and hyperoxia-exposed groups. There was no effect of sex (p_S) or hyperoxia exposure (p_H) on nephron number (A). Nephron density was significantly increased in females compared to males (p_S), with no effect of hyperoxia exposure (B).

Renal corpuscle volume: At P56, renal corpuscle volume was significantly greater (p=0.03) in hyperoxia-exposed kidneys compared to controls (Figure 7A). There was no difference in renal corpuscle volume between males and females. There was a significant inverse linear correlation between renal corpuscle volume and nephron number, within both the control (r^2=0.57, p=0.002) and hyperoxia-exposed (r^2=0.51, p=0.004) groups (Figure 7B). There was a strong trend (p=0.08) for a difference in the slopes of the regression lines between the control and hyperoxia groups, such that for a decline of 1000 nephrons, renal corpuscle volume was increased by 0.20 ± 0.00 mm^3 in control animals, and 0.41 ± 0.12 mm^3 in hyperoxia-exposed animals (Figure 7B).

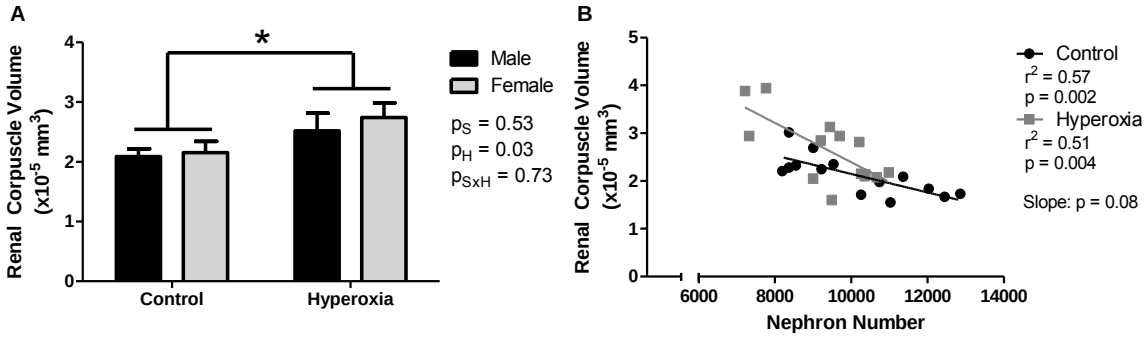

Figure 7: Renal corpuscle volume (**A**) in males (black) and females (grey) in control and hyperoxia-exposed groups. There was a significant effect of hyperoxia exposure (p_H) on renal corpuscle volume, with higher volumes evident in the hyperoxia-exposed animals (A). Linear regression analyses of the correlation between renal corpuscle volume and nephron number (**B**) in control (black) and hyperoxia-exposed (grey) animals. There is a significant inverse correlation within both the control and hyperoxia groups. *p<0.05 control versus hyperoxia.

DISCUSSION

In this study, exposure to hyperoxic gas during the period of postnatal nephrogenesis did not lead to any overt adverse effects on renal development, with no detectable evidence of abnormal renal morphology, or alterations in glomerular size or maturation in the early postnatal period. By early adulthood, however, even though kidney size and nephron number were normal, glomerular hypertrophy had developed in the hyperoxia-exposed kidneys; this may have long-term adverse implications for renal health in that glomerular hypertrophy is strongly linked to the progression of renal disease.

Hyperoxia does not appear to affect nephrogenesis

Given the adverse effects of hyperoxia in other organ systems (31), it was considered likely that hyperoxic exposure in the neonate would also impair nephrogenesis. We hypothesised that the development of glomeruli would be adversely affected as high oxygen concentrations have been shown to impair vascular development, likely due to altered vascular endothelial growth factor (VEGF) expression (7, 20); VEGF is a growth factor essential to glomerular capillary growth and development (4). Furthermore, an increase in oxygen concentration has previously been postulated to trigger the cessation of nephrogenesis in the mouse kidney, with a significant change in the expression profile of genes involved in nephrogenesis evident immediately following birth (2). Contrary to our hypothesis, however, we found no overt evidence to suggest that exposure to hyperoxia adversely impacts renal development in the early postnatal period (P7), at a time when nephrogenesis is ongoing in the mouse; kidney morphology was normal in the hyperoxia-exposed kidneys, and glomerular size and maturation were not different compared to controls.

In adulthood, nephron number was not different between male and female mice; however, nephron density was significantly greater in females which may be explained by their reduced kidney size in adulthood. The results of previous studies investigating sex differences in the C57BL/6 mouse model, with regards to nephron endowment, have to date been inconclusive (13, 24).

In accordance with our finding of no impairment of renal development at P7, nephron number in early adulthood (P56) was also unaffected by neonatal hyperoxia. This is an interesting finding given that body weight was significantly reduced in the hyperoxia-exposed offspring at P7; in general, the number of nephrons formed in the kidney directly correlates with body growth (27). Nephrogenesis in the mouse commences in mid-gestation, and continues during the first 1-2 weeks postnatally. In our study, since the hyperoxic insult was initiated at birth, it was only postnatal growth that was reduced. The short duration of the hyperoxic exposure may therefore account for the absence of any significant reductions in nephron number in the hyperoxia-exposed kidneys.

Contrary to our findings, others have found a 25% reduction in nephron endowment in 25-35 week old rats that were exposed to hyperoxia during the period of postnatal nephrogenesis (41). The disparity in results between studies may be explained by differences in the concentration of hyperoxic gas used, with 65% O_2 in the current study, and 80% O_2 being used in the previous study (41). Certainly, elevated concentrations of oxygen are known to cause increases in oxidative stress (5, 39). Hence, it is likely that any deleterious effects of hyperoxia on nephrogenesis may manifest due to oxidative stress when oxygen concentrations become markedly elevated. Alternatively, the difference in results may relate to the age of the animals at examination. In the previous study (41), nephron number was assessed only at 25-35 weeks of age (compared to 8 weeks of age in the present study). Therefore, the possibility of nephron loss later in adulthood, following the early life hyperoxia insult, cannot be discounted.

Induction of glomerular hypertrophy by adulthood

The major finding of our study was the induction of glomerular hypertrophy in adulthood as a result of early life exposure to hyperoxia; renal corpuscle volume (indicative of glomerular size) was significantly greater in the hyperoxia-exposed animals than in the controls at P56. This finding has important clinical implications given that glomerular hypertrophy is strongly linked to the pathogenesis of renal disease. Indeed, renal corpuscle volume has been shown to be increased in a large number of conditions such as unilateral renal agenesis, obesity-related glomerulopathy, hypertension, oligomeganephronia, diabetes mellitus, and focal segmental glomerulosclerosis (15, 27).

Glomerular hypertrophy often develops as a compensatory response to a reduction in the functional reserve of nephrons, whereby the filtration surface area expands in order to preserve an adequate glomerular filtration rate. However, this is not always the case; glomerular hypertrophy can develop even when nephron endowment is considered to be normal (as seen in the present study) (22, 42). The proliferation of glomerular cells, macrophage infiltration, and accumulation of extracellular matrix following glomerular hypertrophy all contribute to glomerulosclerosis (23). In this way, the compensatory mechanisms that initially preserved glomerular filtration rate ultimately result in irreversible renal injury, and nephron loss (23, 27).

In the present study, there was a significant inverse correlation between nephron number and renal corpuscle volume within both the hyperoxia-exposed and control groups. It should be noted, however, that although nephron endowment was within the normal range, there was a difference in the slope of the regression lines (nearing statistical significance; $p=0.08$) whereby the extent of glomerular enlargement in the hyperoxia-exposed animals, in response to a reduction in nephron number, was much greater than in the controls. The mechanisms leading to this exaggerated glomerular enlargement are unknown. In future studies it would be important to investigate potential mechanisms that may have adversely impacted the functional capacity of the kidney and thus initiated the glomerular hypertrophy in our model. Hypertension, for example, has previously been linked to increased glomerular size, both dependent (14, 16) and independent (17) of nephron number. Notably, in the study by Yzydorczyk et al. (41), neonatal hyperoxia led to long-standing hypertension in male and female rats that was evident from 7-9 weeks of age. Associated with the hypertension was evidence of vascular dysfunction and microvascular rarefaction (41). Whether blood pressure is elevated and/or vascular function is affected in our model requires further investigation.

Conclusions: In a neonatal mouse model with ongoing postnatal nephrogenesis, we have shown that exposure to 65% oxygen during the early postnatal period does not cause immediate changes in renal structure. In the adult kidney, however, early life exposure to hyperoxia was associated with significant glomerular enlargement. Glomerular hypertrophy is a key antecedent of nephron loss and renal disease. The presence of enlarged glomeruli in the young adult kidney is concerning and may have implications for

the long-term renal health of preterm survivors who were exposed to hyperoxic gas during the neonatal period.

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CHAPTER SIX

EFFECTS OF IBUPROFEN TREATMENT ON THE DEVELOPING PRETERM BABOON KIDNEY

CHAPTER SIX DECLARATION

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

Nature of contribution	Extent of contribution (%)
Performed majority of kidney analyses, all of the data analysis, and wrote the manuscript	60%

The following co-authors contributed to the work.

Name	Nature of contribution	Extent of contribution (%) For student co-authors only
Lina Gubhaju	Performed some kidney analyses	5%
Bradley Yoder, Donald McCurnin, Steven Seidner, Ronald Clyman, M. Jane Black	Involved in the design of experiments, performed animal studies, collected physiology data, and assisted in editing the manuscript	35%

Candidate's Signature [redacted] **Date** 30/04/12

Declaration by co-authors

The undersigned hereby certify that:

(43) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.

(44) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(45) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(46) there are no other authors of the publication according to these criteria;

(47) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(48) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) **Department of Anatomy & Developmental Biology, Monash University**

	Signature	Date
Bradley Yoder	[redacted]	25 April 2012
Donald McCurnin	[redacted]	25 April 2012
Steven Seidner	[redacted]	4/25/12
Lina Gubhaju	[redacted]	24/4/2012
Ronald Clyman	[redacted]	30/4/12
M. Jane Black	[redacted]	24/4/12

EFFECTS OF IBUPROFEN TREATMENT ON THE DEVELOPING PRETERM BABOON KIDNEY

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ABSTRACT

Preterm neonates are commonly exposed postnatally to pharmacological treatments for a patent ductus arteriosus. Exposure of the developing kidney to nephrotoxic medications may adversely impact renal development. This study aimed to determine the effect of early postnatal ibuprofen treatment, both alone, and in combination with a nitric oxide synthase inhibitor (NOSi), on renal development and morphology. Baboon neonates were delivered prematurely at 125d gestation (term = 185d) and were euthanized at birth or postnatal day 6. Neonates were divided into four groups: 125d gestational controls (n=8), Untreated (n=8), Ibuprofen (n=6), and Ibuprofen+NOSi (n=4). Animals in the Ibuprofen and Ibuprofen+NOSi groups received 5 doses of ibuprofen, with the Ibuprofen+NOSi animals additionally administered a NOS inhibitor (L-NMMA). There was no difference among groups in body weight, kidney weight or glomerular generation number. Nephrogenic zone width was significantly reduced in the Ibuprofen group (123.5 ± 7.4 μm) compared to the 125d gestational control (176.1 ± 6.9 μm) and Untreated animals (169.7 ± 78.8 μm). In the Ibu+NOSi group, nephrogenic zone width averaged 152.7 ± 3.9 μm which was not significantly different to any other group. Morphologically abnormal glomeruli were present at a range of 0.0% to 22.9% in the Untreated group, 0.0% to 6.1% in the Ibuprofen group, and 0.0% to 1.4% in the Ibu+NOSi group. In conclusion, early postnatal ibuprofen exposure is associated with a reduced nephrogenic zone width, which may suggest the early cessation of nephrogenesis following treatment. Ultimately, this may impact the number of nephrons formed in the preterm kidney.

INTRODUCTION

Nephrogenesis is normally completed *in utero* between 32 and 36 weeks gestation (13). Therefore, infants born extremely preterm (< 28 weeks gestation) are born at a time when nephrogenesis is still ongoing. Although it has been shown that nephrogenesis continues postnatally after preterm birth (10, 31), there is evidence to suggest that it may be impaired with previous reports of reduced glomerular generation formation (24), accelerated postnatal maturation (31), and the presence of morphologically abnormal glomeruli in the outer renal cortex of the preterm kidney (10, 24, 31, 32). These glomeruli are cystic in appearance, exhibiting an enlarged Bowman's space and shrunken glomerular tuft (10, 31, 32). Through previous studies in a baboon model of preterm birth, we have demonstrated that the proportion of abnormal glomeruli varies considerably between neonates (10, 32), suggesting that the morphological abnormalities may not be attributed to preterm birth *per se*, but rather to factors in the postnatal clinical course of the preterm neonate (which varies with each neonate).

Exposure to medications commonly occurs during the period of postnatal nephrogenesis in the preterm neonate. Non-steroidal anti-inflammatory drugs (NSAIDs) are used in the treatment of a patent ductus arteriosus (PDA), a condition affecting up to 70% of extremely preterm neonates (11). NSAIDs, such as ibuprofen, act by preventing prostaglandin synthesis via the inhibition of cyclooxygenase (COX) enzyme activity (36). Following NSAID treatment 30% of infants fail to close their PDA, or after an initially successful closure the PDA will reopen (8, 28). This failure to respond to treatment has been attributed to a compensatory increase in vasodilatory nitric oxide production (2, 3, 29). Recent studies in preterm baboon and human infants demonstrate that treatment of a PDA with a combination of NSAIDs and nitric oxide synthase inhibitors significantly increases the success of ductus closure compared to NSAID treatment alone (16, 26).

Importantly, there is experimental evidence to suggest that exposure to NSAIDs can adversely impact nephrogenesis. Komhoff *et al*. (19) and Olliges *et al*. (23) recently determined that NSAID exposure led to a significantly reduced cortical mass, reduced glomerular density, and reduced glomerular and tubular volumes in mice models of NSAID exposure during the period of postnatal renal development. Similarly, Kent *et al*.

(17, 18) observed a significantly reduced glomerular density and also renal injury in neonatal rats with early postnatal NSAID exposure; however, there was no effect on total nephron endowment. Of particular importance, in human infants exposed *in utero* to NSAIDs, changes in renal morphology have been described (15, 33) which are similar to the glomerulocystic changes observed in the preterm baboon kidney. NOS inhibitors elicit a similar physiological response as NSAIDS, such as reduction in renal blood flow and urine output (27); however, the effects on nephrogenesis have not been previously investigated. Therefore, the aim of this study was to determine the effects of ibuprofen treatment on renal development and morphology in the preterm baboon kidney, both alone, and in combination with a NOS inhibitor.

METHODS

Animal Care and Treatment Groups

All animal studies were performed at the Southwest Foundation for Biomedical Research (San Antonio, Texas, USA), and were approved by the institutional animal care and use committee. Baboon neonates were delivered prematurely by caesarean section at 125 days gestation (term = 185 days), a time-point equivalent to 27 weeks gestation in humans. Animals in the gestational (fetal) control group (125d; n = 8) were euthanized at delivery. The remainder of animals were cared for in a primate intensive care nursery and were euthanized on postnatal day 6 (144 hours after delivery), with the exception of one animal in the Ibu+NOSi group which was euthanized on postnatal day 5 (120 hours after delivery).

Preterm baboon neonates received one of three treatment protocols, as have been previously described (26, 35): 1) No treatment (Untreated; n = 8); 2) Ibuprofen alone (Ibuprofen; n = 6); 3) Ibuprofen plus nitric oxide synthase inhibitor (Ibu+NOSi; n = 4).

In the Ibuprofen group, ibuprofen lysine (Farmacon; Westport, CT, USA) was administered intravenously at 10 mg/kg (over 20 min) at 24 hours of age, followed by 5 mg/kg at 48, 72, 96, and 120 hours of age. This dosing regime was based on the recommended dosage of ibuprofen for the treatment of a patent ductus arteriosus in human preterm infants (22). In the Ibu+NOSi group, ibuprofen was given in combination with the nitric oxide synthase inhibitor N^{G}-monomethyl-$_{L}$-arginine (L-NMMA). Treatment with L-NMMA (Calbiochem; San Diego, CA, USA) was initiated at 50 hours after delivery, and was continuously infused at a rate of 20 mg/kg/h until the time of necropsy.

Physiological Measurements

Echocardiographic assessment of ductal patency was performed daily using an 8-mHz transducer interfaced with a Biosound AU3 echocardiographic system (Genoa, Italy). Animals were instrumented with an umbilical arterial catheter which enabled measurement of blood pressure, and the administration of fluid requirements. Fluid intake and urine output was continuously recorded over the 6 days of life. Mean blood

pressure, at 12, 24, 48, 96, and 120 hours of life, was determined by averaging three measured values of systolic and diastolic blood pressure (within a 4-6 hour period) around the defined time-point.

In the assessment of blood pressure, infusion rates of L-NMMA were reduced by 25% if mean systemic blood pressure was consistently > 47 mmHg. In cases of significant hypotension (defined as a mean blood pressure of < 25 mmHg, accompanied by either increasing base deficit or decreasing urine output) volume supplementation was first initiated (10–20 ml/kg, administered at least twice over a 1 hour period) followed by the use of inotropic support. Dopamine was initially administered at a rate of 4–6 µg/kg/min, and further increased to a maximum rate of 20 µg/kg/min. If mean blood pressure failed to respond to volume and inotropic drugs within 2–4 hours, then hydrocortisone (Soul-Cortef, Pharmacia & Upjohn; Kalamazoo, MI, U.S.A.) at a dose of 0.5–1.0 mg/kg was administered at 6 hourly intervals until either mean blood pressure increased to > 28 mm Hg or a maximum of four doses of hydrocortisone were received.

Tissue Collection and Processing

Kidneys collected at necropsy from animals in the Ibuprofen and Ibu+NOSi groups, and from five animals from the Untreated group, were embedded in Tissue-Tek OCT compound and snap frozen in liquid nitrogen. Kidney tissue from the 125d gestational control group, and from three animals in the Untreated group, was formalin-fixed and embedded in paraffin. There was no significant difference observed in any measured parameters between the formalin-fixed paraffin-embedded tissue and the frozen tissue within the Untreated group.

Kidneys were halved along the coronal axis prior to embedding, and sectioned at a similarly central region for each kidney. 5-7 µm sections were stained with haematoxylin and eosin. In all assessments of renal development and morphology, the researcher was blinded to the experimental grouping of the animals.

Assessment of Renal Development and Morphology

Nephrogenic Zone Width

The width of the nephrogenic zone, defined as the area in the outer renal cortex exhibiting developing glomerular structures in the form of comma and S-shaped bodies, was measured using image analysis software (Image Pro Plus v. 6.0 for Windows; Media Cybernetics, Silver Spring, MD, USA). This method has previously been utilised to assess renal maturity in both human (5, 31) and baboon (10) fetal and neonatal kidneys. One stained section from each kidney was viewed at 200X magnification and the width of the nephrogenic zone was measured in four separate regions. An average nephrogenic zone width was determined for each kidney.

Glomerular Generation Number

The medullary ray glomerular generation counting method was utilised to estimate the number of glomerular generations formed within the kidney. This method has been validated by Hinchliffe and colleagues (12), and also utilised in previous studies to assess renal maturity in preterm human (6, 24, 31) and baboon neonates (10, 32). In one complete section from each kidney, five clearly distinguishable medullary rays from separate regions of the section were identified and the number of mature glomeruli along one side of the medullary ray was counted. An average number of glomerular generations was determined for each kidney.

Glomerular Morphology

One complete kidney section from each kidney was systematically sampled at a step length of 1 mm. At each field of view, the numbers of normal and abnormal mature glomeruli were recorded. Glomeruli were classified as abnormal if they exhibited a grossly enlarged Bowman's space and a shrunken glomerular tuft (10, 31, 32). The percentage of abnormal glomeruli per kidney was determined.

Statistical Analysis

Data were analysed using GraphPad Prism software (v5.03 for Windows) and Intercooled Stata (v8.0 for Windows) and graphed as the mean ± the standard error of the mean (SEM). Analysis of physiological parameters (fluid intake, urine output, and blood pressure) was undertaken using a two-way repeated measures analysis of variance (ANOVA), with the factors T (Treatment), A (Age) and TxA (Interaction). To determine differences between groups at individual time-points, the two-way ANOVA was followed by a Bonferroni post-hoc test. To determine differences in categorical variables among groups (including ductus closure, dopamine administration, hydrocortisone administration, oliguria, and a high (> 5%) or low (< 5%) percentage of abnormal glomeruli) a Fisher's exact test was performed. All other statistical comparisons among groups (body and kidney weights, assessments of renal morphology) were performed using a one-way ANOVA followed by a Bonferroni post-hoc test. Statistical significance was accepted at the level of $p \leq 0.05$.

RESULTS

Body Weight and Kidney Weight

Birth weight, necropsy weight, kidney weight, and kidney weight to body (necropsy) weight ratios for each group are shown in Table 1. There was no significant difference between any of the gestational control (125d) or preterm (Untreated, Ibuprofen, Ibu+NOSi) groups in any parameter of body and kidney weight.

Table 1: Body and kidney weights of the gestational control (125d) and preterm (Untreated, Ibuprofen, and Ibu+NOSi) baboons.

	125d (n=8)	**Untreated (n=8)**	**Ibuprofen (n=6)**	**Ibu+NOSi (n=4)**
Birth Weight (g)	382.9 ± 20.4	381.9 ± 10.7	388.8 ± 17.7	395.3 ± 16.4
Necropsy Weight (g)	382.9 ± 20.4	377.1 ± 15.8	352.8 ± 17.1	417.8 ± 42.2
Combined Kidney Weight (g)	2.9 ± 0.2	3.2 ± 0.1	3.0 ± 0.3	3.4 ± 0.3
Kidney Weight to Body Weight Ratio (g/kg)	7.9 ± 0.7	8.5 ± 0.3	8.3 ± 0.6	8.4 ± 0.8

Values are given as the mean ± SEM.

Fluid Intake and Urine Output

Fluid intake was reduced significantly with increasing postnatal age (Figure 1A). There was no effect of treatment on fluid intake, but a significant interaction effect between postnatal age and treatment was evident.

There was no significant effect of treatment on urine output over the 6 days of life (Figure 1B). Overall, there was a significant effect of postnatal age on urine output, and a significant interaction effect between postnatal age and treatment. Urine output was significantly reduced in the Ibu+NOSi group compared to the Untreated group at both 96 and 120 hours of age.

Oliguria (urine output < 1 ml/kg/hr) was exhibited in 3/8 Untreated animals, 1/6 Ibuprofen animals, and 1/4 Ibu+NOSi animals, with no association between treatment group and oliguria (p = 0.80).

Ductus Closure and Blood Pressure

There was a strong association between treatment and ductus closure (p=0.005). In all Ibuprofen and Ibu+NOSi treated animals, the ductus was closed on days 2-3 of life and remained closed until necropsy. Two of the Untreated animals achieved ductus closure, which occurred on day 4 of life. In the remainder of Untreated animals, the ductus remained open throughout the 6 day study period.

Overall, there was a significant effect of postnatal age on mean blood pressure (Figure 1C). There was also a strong trend towards an effect of treatment on mean blood pressure, however this did not quite reach statistical significance (p=0.08).

There was a significant association between treatment group and dopamine administration (p=0.002), where it was required in 7/8 Untreated, 0/6 Ibuprofen, and 2/4 Ibu+NOSi animals. Similarly, hydrocortisone administration was most common in the Untreated group where it was required in 4/8 animals, and was not administered to any of the Ibuprofen or Ibu+NOSi animals (p=0.05).

Nephrogenic Zone Width

As shown in Figure 2, the width of the nephrogenic zone averaged 176.1 ± 6.9 μm in the 125d gestational control group, and was not different from the Untreated group at postnatal day 6 (169.7 ± 8.8 μm). Ibuprofen treatment alone significantly reduced nephrogenic zone width by 30% compared to the 125d group, and 27% compared to the Untreated group, with a mean of 123.5 ± 5.8 μm. Ibu+NOSi animals had a mean nephrogenic zone width of 152.7 ± 3.9 μm which was not different to any other group.

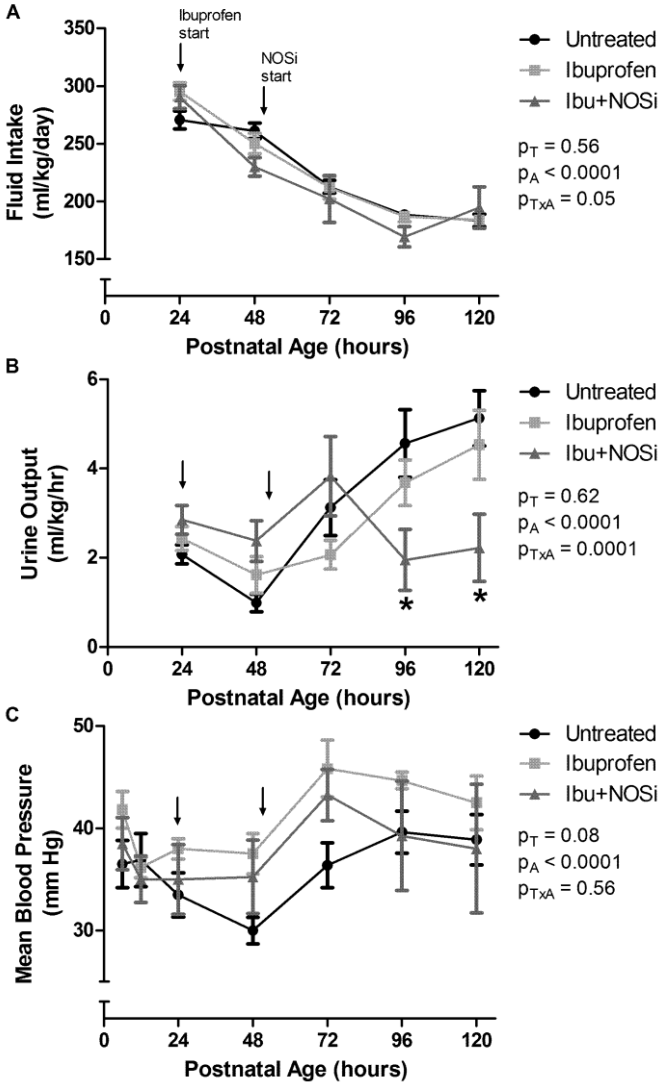

Figure 1: Fluid intake (**A**), urine output (**B**), and mean blood pressure (**C**), up to 120 hours of life, in preterm baboons in the Untreated, Ibuprofen, and Ibu+NOSi groups. Arrows mark the starting point for Ibuprofen administration (24 hours; Ibuprofen and Ibu+NOSi groups) and NOSi administration (50 hours; Ibu+NOSi group). *p < 0.05 Ibu+NOSi *versus* Untreated. Data is presented as the mean ± SEM.

Glomerular Generation Number

In the 125d gestational control group, the number of glomerular generations averaged 6.8 ± 0.2. Similarly, in the Untreated group at postnatal day 6, mean glomerular generation number was 6.4 ± 0.1. There was no effect of Ibuprofen (6.6 ± 0.1) or Ibu+NOSi (6.7 ± 0.2) treatment on glomerular generation number.

Glomerular Morphology

Morphologically abnormal glomeruli, with an enlarged Bowman's space and shrunken glomerular tuft (Figure 3), were commonly observed in the outer renal cortex of the preterm kidneys at postnatal day 6, whereas the percentage of abnormal glomeruli was negligible (ranging from 0.0% to 2.4%) in the 125d gestational controls (Figure 4). In the Untreated group, the percentage of abnormal glomeruli was markedly variable among individuals, ranging from 0.0% to 22.9%. Similarly, in the Ibuprofen group the range was 0.0% to 6.1%. In the Ibu+NOSi animals, however, the percentage of abnormal glomeruli ranged from 0.0% to 1.4%. The percentage of abnormal glomeruli was not statistically different among the four groups.

Five animals (Untreated: n=4, Ibuprofen: n=1) had a percentage of abnormal glomeruli greater than 5% (Figure 4). At 24 hours of age, mean blood pressure was significantly reduced in the group of animals with > 5% of glomeruli, compared to those with a low percentage of abnormal glomeruli ($p = 0.01$). There was also a trend for decreased urine output at 48 hours of age in those animals with a high percentage of abnormal glomeruli ($p=0.06$). Two of the five animals with > 5% abnormal glomeruli, and 4/13 with < 5% abnormal glomeruli exhibited oliguria during the study period ($p = 1.00$).

Four of the five animals with > 5% abnormal glomeruli were treated with dopamine; this was not significantly different to the group of animals with a low percentage of abnormal glomeruli where 5/13 animals required dopamine treatment ($p = 0.29$). The number of baboons that required hydrocortisone treatment was significantly greater ($p = 0.04$) in the group of animals with > 5% abnormal glomeruli (3/5 animals; 1/3 receiving 1 dose, and 2/3 receiving 3 doses) compared to the animals that exhibited a low percentage of abnormal glomeruli (1/13 animals; receiving 1 dose).

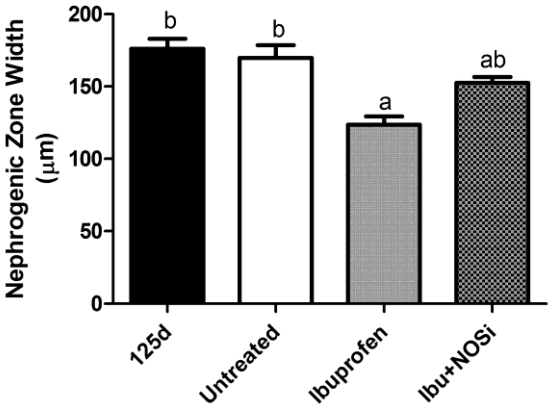

Figure 2: The width of the nephrogenic zone in the kidneys of gestational control baboons (125d) and in preterm baboons (Untreated, Ibuprofen, and Ibu+NOSi) analysed at postnatal day 6. Significant differences among groups ($p < 0.05$) are indicated by the letters; a is different from b, but not from ab. Data is presented as the mean ± SEM.

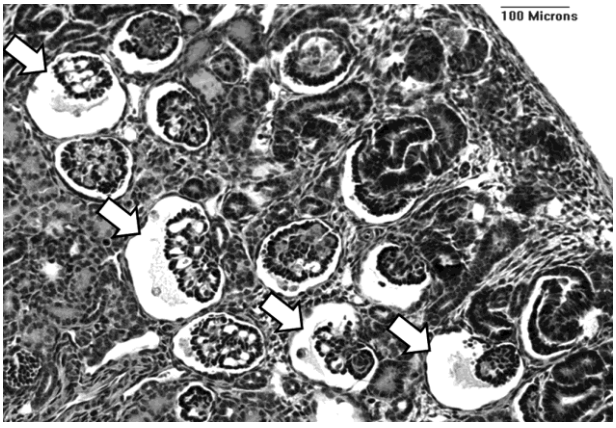

Figure 3: Representative photomicrograph of abnormal glomeruli (arrows), exhibiting an enlarged Bowman's space and shrunken glomerular tuft, in the outer renal cortex of a preterm baboon kidney (Untreated).

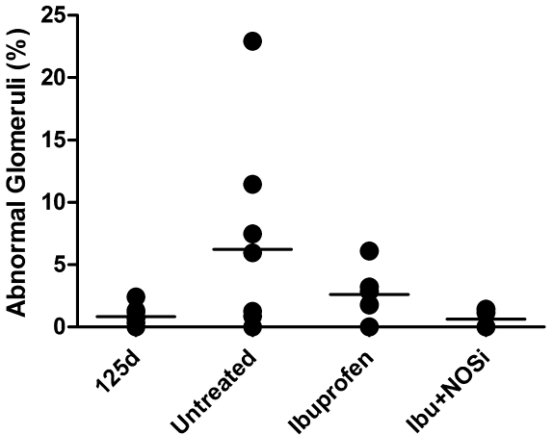

Figure 4: The percentage of morphologically abnormal glomeruli in the kidneys of gestational control baboons (125d) and in preterm baboons (Untreated, Ibuprofen and Ibu+NOSi) analysed at postnatal day 6.

DISCUSSION

Using a baboon model of preterm birth, where nephrogenesis is still ongoing postnatally, we have demonstrated that early postnatal exposure to the non-steroidal anti-inflammatory drug ibuprofen does not influence glomerular generation number or the percentage of morphologically abnormal glomeruli at postnatal day 6. Of concern, however, ibuprofen treatment led to a significantly reduced nephrogenic zone width, which may be indicative of the early cessation of nephrogenesis.

Pharmacological treatments for PDA have significant renal side-effects in the preterm neonate. NSAID treatment increases renal vascular resistance, reduces renal blood flow (14, 30), decreases glomerular filtration rate (1, 22), and is an independent risk factor for the development of acute renal failure (4). Similarly, nitric oxide synthase inhibition has been shown to significantly reduce renal blood flow, glomerular filtration rate, and alter tubular function in neonatal animals (27). Furthermore, combination NOS inhibitor and NSAID treatment has been shown to produce significant renal side-effects (such as increased serum creatinine) in human preterm neonates (16). In the current study, fluid intakes were reduced in all preterm neonates over the course of the study (figure 1), likely in response to improved cardiovascular function and reductions in insensible fluid loss with increasing postnatal age. Urine output was significantly lower in the Ibu+NOSi group compared to the Untreated group at the 96 and 120 hour time-points, indicative that renal function was somewhat affected by the treatment; however, there was no effect of Ibuprofen or Ibu+NOSi treatment on the number of animals that presented with oliguria during the study period.

As expected, there was a strong association between treatment and ductus closure, with all animals in the Ibuprofen and Ibu+NOSi groups achieving ductus closure by postnatal day 4, compared to just two animals in the Untreated group. The effect of treatment on mean blood pressure did not quite reach statistical significance (figure 1); however, dopamine and hydrocortisone treatment for hypotension was most common within the Untreated group. The lack of a substantial effect of treatment on blood pressure in this study may be explained by the strict maintenance of fluid requirements, and the adjustment of NOS inhibitor, dopamine and hydrocortisone dosage in cases of significant

hypo- and hypertension. Hence, it is to be noted that exposure to dopamine and hydrocortisone treatments are confounding variables which could not be controlled for in this study.

Importantly, the results indicate that early postnatal exposure to ibuprofen leads to a significant reduction in nephrogenic zone width (figure 2). The width of the nephrogenic zone measured just 123.5 ± 5.8 µm in the Ibuprofen group, reflecting a 27% reduction in width compared to the Untreated animals. A reduced nephrogenic zone width is suggestive of either an early cessation of nephrogenesis and/or an increase in renal maturation following treatment. Such an effect on nephrogenesis has the potential to result in a nephron deficit, which in turn has long-term consequences for renal health (9). The finding of a reduced nephrogenic zone width is in accordance with previous studies in rodent models, where exposure to NSAIDs during the period of postnatal nephrogenesis (a longer time period of exposure than in the current study) resulted in impaired nephrogenesis (19) and also renal injury (18). These effects may be mediated via the inhibition of COX enzyme activity within the developing kidney. COX expression has been shown to be essential for renal development, with COX-2 (but not COX-1) knock-out mice exhibiting severe renal dysplasia at birth (21). In addition, adult rats following both prenatal and early postnatal exposure to a COX-2 inhibitor exhibited a significant nephron deficit, with associated glomerular hypertrophy and glomerulosclerosis (25). The exact role of COX-2 in renal development, however, has not been fully elucidated.

Interestingly, nephrogenic zone width in the group of animals that received the combined ibuprofen and NOS inhibitor treatment was not significantly reduced compared to the Untreated control group. This result suggests that the inhibition of NO synthesis may ameliorate the effects of ibuprofen treatment on width of the nephrogenic zone. The reasons for this are unknown; however, there is known to be much cross-talk between the prostaglandin and nitric oxide pathways. In some tissues, for example, prostaglandin inhibition leads to a significant increase in nitric oxide synthase expression and nitric oxide production (29, 34). Increased nitric oxide levels have been shown to inhibit cellular adhesion, extracellular matrix synthesis, and proliferation of cultured mesangial cells (7), and also to promote glomerular apoptosis (20). An analysis of prostaglandin and NO levels within the preterm kidney, particularly within the developing outer renal

cortex, would help to elucidate the mechanisms underlying the response of nephrogenesis to NSAID and NOS inhibitor treatment.

Despite the reduced nephrogenic zone width, glomerular generation number was not affected; this likely relates to the early timing of examination at just five days following the onset of ibuprofen treatment. Furthermore, we would expect the potentially adverse effects of ibuprofen exposure may be of short duration given that the recommended treatment for patent ductus arteriosus is only three doses of either indomethacin or ibuprofen, at 12 - 24 hour intervals (22). Therefore, it is possible that any adverse effect NSAID exposure has on the kidney may be short-lived and therefore would not significantly influence final nephron endowment. Indeed Kent *et al*. (17), in a stereological assessment of nephron endowment following *in vivo* NSAID exposure, demonstrated in a neonatal rat model that total nephron endowment was not altered following extended postnatal exposure to NSAIDs. In that study, the NSAIDs were administered over a period equivalent to 24-30 weeks gestation in humans (17), a much longer time period of exposure than in the current study. An examination of the baboon kidneys at a later postnatal time-point, after the treatments and nephrogenesis have ceased, would be required in order to fully describe the long-term effects of NSAID exposure on renal development.

Consistent with previous studies in this model (10, 32), and also in the human preterm neonate (31), morphologically abnormal glomeruli were commonly present in the outer renal cortex of the preterm kidneys (figure 3). The proportion of abnormal glomeruli was highly variable; there was no statistically significant difference in the percentage of abnormal glomeruli among treatment groups (figure 4). In the Untreated animals, the percentage of abnormal glomeruli ranged from as low as 0% to as high as 22.9% (a very abnormal kidney). Importantly, our findings demonstrate that ibuprofen treatment does not lead to the glomerular abnormalities associated with preterm birth. In the ibuprofen-treated animals, the number of abnormal glomeruli ranged from 0-6%; the animals that received the combined ibuprofen and NOS inhibitor treatment exhibited the lowest percentage of abnormal glomeruli, at 1.4% or less per kidney. Although glomerular abnormalities have been described in the human kidney following antenatal NSAID exposure (15, 33), the results of this study clearly indicate that early postnatal exposure

to ibuprofen is certainly not the cause of the abnormal glomeruli commonly observed in the outer renal cortex of the preterm kidney.

The cause of the glomerular abnormalities, therefore, remains unknown. In the current study, animals with a high percentage (> 5%) of abnormal glomeruli had significantly lower blood pressure at 24 hours of age, and also a trend towards a reduced urine output at 48 hours of age, compared to those animals with a low percentage of abnormal glomeruli. Furthermore, there was a significant association between a high percentage of abnormal glomeruli and a requirement for hydrocortisone treatment. From these results it may be speculated that changes in blood pressure and perhaps renal blood flow may be involved in the formation of the morphologically abnormal glomeruli in the preterm kidney. An analysis of renal blood flow changes following preterm birth would be required in the future, however, in order to definitively establish whether this is the cause of the glomerular abnormalities.

Alternatively, it may be the hydrocortisone treatment itself (administered following persistent hypotension in baboon neonates that did not respond to dopamine treatment) that may be linked to the formation of abnormal glomeruli. It is to be noted, however, that we have previously shown in a separate cohort of animals that dopamine and hydrocortisone administration were not associated with a high percentage of abnormal glomeruli (32); in a group of 12 baboons delivered preterm at 125d gestation, the percentage of abnormal glomeruli per kidney ranged from 0.00% to 16.03% in baboons not exposed to dopamine (mean: 8.06 ± 2.7%), and from 0.55% to 2.56% in baboons that were exposed (mean: 1.3 ± 0.4%). When hydrocortisone was additionally administered, the percentage of abnormal glomeruli was very low (1.14%) (32).

In conclusion, early postnatal ibuprofen treatment does not influence the percentage of morphologically abnormal glomeruli in the preterm kidney. Of concern, however, ibuprofen treatment led to a significantly reduced nephrogenic zone width, which may be indicative of the early cessation of nephrogenesis. Ultimately, this may impact on the number of nephrons formed in the preterm kidney.

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CHAPTER SEVEN

DISCUSSION AND CONCLUSIONS

DISCUSSION AND CONCLUSIONS

Through the comprehensive assessments of renal development and function in the preterm neonatal kidney reported in this thesis, it has been shown that although nephrogenesis is ongoing after preterm birth, renal development may be impaired. In particular, the structural development of the kidney is accelerated postnatally (with a reduced nephrogenic zone width) and glomeruli are significantly enlarged (possibly following initial glomerular capillary dilation). Of particular concern, in some preterm neonates glomerular morphology was abnormal in a high proportion of glomeruli. Importantly, it was determined that factors commonly involved in the postnatal care of the preterm neonate may affect renal development and/or long-term renal morphology; ibuprofen treatment in the early postnatal period may lead to the early cessation of nephrogenesis, and exposure to hyperoxia during the neonatal period was linked to glomerular hypertrophy in adulthood. It was also shown that the preterm kidney is functionally immature throughout the first month of life, and findings of severe proteinuria and high urinary NGAL levels in some infants were likely indicative of postnatal renal injury.

Overall, the results of the studies reported in this thesis suggest that preterm birth may adversely influence the nephrogenic potential of the neonate, therefore resulting in a deficit of functional nephrons at the beginning of life. Findings of capillary dilation in the immediate postnatal period, as well as significant glomerular hypertrophy in the adult kidney following neonatal hyperoxia exposure, are also of particular concern; glomerular hypertrophy (and the subsequent development of glomerulosclerosis and eventual nephron loss) is a key antecedent of renal disease. Overall, these findings suggest there may be significant adverse consequences to the lifelong renal health of individuals born preterm.

7.1 EFFECTS OF PRETERM BIRTH ON RENAL MORPHOLOGY AND FUNCTION

At the commencement of this research, it was initially hypothesised that preterm birth would impair nephrogenesis, be causative of renal injury, and also impair renal function in

the neonate. Ultimately, these changes were proposed to lead to a nephron deficit and thus increase the risk that the preterm neonate would develop renal disease later in life. Through the series of studies reported in this thesis, it was shown that preterm birth does affect postnatal nephrogenesis, with evidence of accelerated renal development in the early postnatal period; this is likely to negatively impact on the number of nephrons formed within the kidney after birth. Both glomerular and tubular function in the early postnatal period were also impaired in comparison to term-born controls. The presence of morphologically abnormal glomeruli and glomerular hypertrophy in the preterm neonatal kidney, in addition to severe proteinuria and high urinary NGAL levels, may be indicative of postnatal renal injury; this will likely result in nephron loss. Hence, these findings support the initial hypothesis that preterm birth results in a deficit of functional nephrons at the beginning of life and this is likely to lead to life-long renal vulnerability.

7.1.1 RENAL MORPHOLOGY

ACCELERATED RENAL DEVELOPMENT

Consistent with previous studies conducted in our laboratory in a preterm baboon model (Gubhaju *et al.*, 2009), including the findings from my Honours project (Sutherland *et al.*, 2009), the results of the current thesis have definitively shown that nephrogenesis is ongoing in the human neonatal kidney following preterm birth (Chapter 3; Sutherland *et al.* (2011)); a clear nephrogenic zone was observed in the outer renal cortex of the preterm kidneys (examined at 2-68 days postnatal age), and the number of glomerular generations was significantly increased compared to gestational controls. Importantly, in assessing the growth trajectory of the preterm kidney in comparison to postconceptional age-matched controls, significant differences in renal morphology were observed which were suggestive of accelerated postnatal renal development. These included a significant reduction in nephrogenic zone width and a reduced percentage of immature vesicle-stage glomeruli, in association with a significant increase in glomerular generation number in the preterm neonatal kidney. As the majority of preterm kidneys assessed in this study had ongoing nephrogenesis, however, it was not possible to accurately determine the effects of accelerated renal development on final glomerular generation number (and thus nephron endowment). It is conceivable that the accelerated postnatal renal

development may ultimately result in the early cessation of nephrogenesis, and thus a reduction in the final number of nephrons formed. This theory is supported by the findings of Rodriguez *et al.* (2004) whereby preterm neonates (in particular those with a history of acute renal failure) were shown to have a significantly reduced number of glomerular generations compared to term-born controls. Additionally, a recently published experimental study demonstrated that a 20% reduction in nephron number occurred following premature delivery at 1-2 days prior to term birth in a preterm mouse model (Stelloh *et al.*, 2012).

GLOMERULAR HYPERTROPHY

In the assessment of renal morphology in the human neonatal kidney (Chapter 3), it was also found that renal corpuscle cross-sectional area was significantly increased in the preterm neonates compared to the gestational controls, which is indicative of glomerular hypertrophy (Sutherland *et al.*, 2011). Similarly, in the lamb kidney following moderate preterm birth (Chapter 4), there was significantly increased glomerular capillary surface area density per renal corpuscle, in comparison to previously published findings in gestational controls (Mitchell *et al.*, 2004). The glomerular capillary surface area density in the preterm lamb kidney had in fact increased to a level after birth that was equivalent to that of term-born lambs, despite a lesser glomerular capillary length density in the preterm animals (likely reflecting the immaturity of the glomeruli); to my knowledge, this is the first study to have examined glomerular capillary growth following preterm birth (Chapter 4). It is probable that increases in glomerular blood flow after birth (described in Section 7.2.2) as well as the increased functional demand placed on the immature kidney (an adequate glomerular capillary surface area is required in order to maintain GFR) may have contributed to the capillary dilation. Importantly, regardless of the cause of the glomerular hypertrophy (and/or capillary dilation) in the preterm neonatal kidney (Chapters 3 and 4), it is indicative of hyperfiltration and if it persists into later life it will likely result in the development of glomerulosclerosis and eventual nephron loss (Kriz and Endlich, 2005; Metcalfe, 2007; Puelles *et al.*, 2011).

MORPHOLOGICALLY ABNORMAL GLOMERULI

Of concern, morphologically abnormal glomeruli, exhibiting a grossly enlarged Bowman's space and shrunken glomerular tuft, were commonly observed in the outer renal cortex of the human preterm neonatal kidney (Chapter 3; Sutherland *et al*. (2011)); this finding is in accordance with other studies in human preterm neonates (Rodriguez *et al.*, 2004), as well as in the preterm baboon model (Gubhaju *et al.*, 2009; Sutherland *et al.*, 2009). Not all preterm neonates were affected, and the percentage of abnormal glomeruli varied considerably between individual neonates (ranging from 0.0% up to 13.7% per kidney). Therefore, it does not appear to be preterm birth *per se* that has led to the glomerular abnormalities, but rather it may be factors involved in the postnatal clinical course of the preterm neonate (that vary between individuals) that underlies their pathogenesis; importantly, I have shown that neither early postnatal hyperoxia exposure (Chapter 5) or ibuprofen treatment (Chapter 6) are the cause.

In all kidneys, the abnormal glomeruli were exclusively localised to the outer renal cortex, indicating that it is the glomeruli formed in the extrauterine environment that are vulnerable (Chapter 3). In support of this idea, abnormal glomeruli were not present in the kidney of lambs that were delivered moderately preterm, at a time point when nephrogenesis was already completed (Chapter 4). From the histological appearance of the abnormal glomeruli (enlarged Bowman's space and shrunken glomerular tuft) it is speculated that these glomeruli are atubular (Marcussen, 1992; Gibson *et al.*, 1996); if this is the case, the affected glomeruli will never be functional. Therefore, a high percentage of abnormal/atubular glomeruli, such as was observed in the kidney of a number of preterm neonates, would markedly impact on the life-long renal functional capacity of individuals born preterm. In future studies, it is important to determine whether the abnormal glomeruli in the preterm kidney are atubular; this could be achieved by assessing serial sections of the abnormal glomeruli.

7.1.2 RENAL FUNCTION

In the assessment of renal function in preterm neonates during the first month of life (Chapter 2), both gestational and postnatal age were shown to be strongly correlated

with indices of glomerular and tubular function; with increasing age, creatinine clearance was significantly increased, and sodium and protein excretion were significantly decreased. These findings are similar to those reported in previous studies of renal function in preterm neonates (Tsukahara *et al.*, 1994; Vieux *et al.*, 2010; Bonsante *et al.*, 2011), and emphasise the importance of renal structural maturity on both glomerular and tubular function in the preterm neonate.

It is unknown, however, whether the impaired renal function (severe proteinuria and/or high urinary NGAL levels) observed in a proportion of the preterm neonates was due to renal immaturity, or whether it was caused by acute kidney injury (Parikh *et al.*, 2010). There were twelve neonates in particular (comprising 9% of the study population) that exhibited pathological proteinuria during the first month of life, with a small subset of these neonates further exhibiting high urinary NGAL levels at corresponding time points. Given that the onset of severe proteinuria was often at day 21 or day 28 of life (not immediately after birth), and given the severity of renal dysfunction in these neonates, it is likely that significant renal injury occurred during the postnatal period. Importantly, acute kidney injury (and proteinuria) in the neonatal period may lead to progressive renal disease (Abitbol *et al.*, 2003; Abbate *et al.*, 2006). It is therefore of extreme importance to assess the utility of potential biomarkers of AKI in preterm neonates, in order to ensure the early diagnosis and treatment of renal injury. In this regard, the findings of this thesis showed that urinary NGAL levels were not a good predictor of renal dysfunction in the preterm neonate, whereby there was no correlation between urinary NGAL levels and the occurrence of pathological proteinuria. Hence, the utility of alternative biomarkers for the diagnosis of renal injury in the preterm neonate need to be explored.

7.2 POTENTIAL CAUSES OF IMPAIRED RENAL DEVELOPMENT AND INJURY

The underlying mechanisms that lead to the accelerated renal development, glomerular hypertrophy, impaired renal function, and the presence of morphologically abnormal glomeruli in the neonatal kidney following preterm birth are currently unknown. From the results of studies of renal function (Chapter 2) and renal morphology (Chapter 3) in the human preterm neonate, however, it is clear that there is great variability in the

severity of renal dysfunction/injury between individual neonates. In this regard, there are a number of factors related to the antenatal and/or postnatal environment of the preterm neonate that are likely to directly influence renal development and function (Figure 7.2), and may also be causative of renal injury in the preterm neonatal kidney.

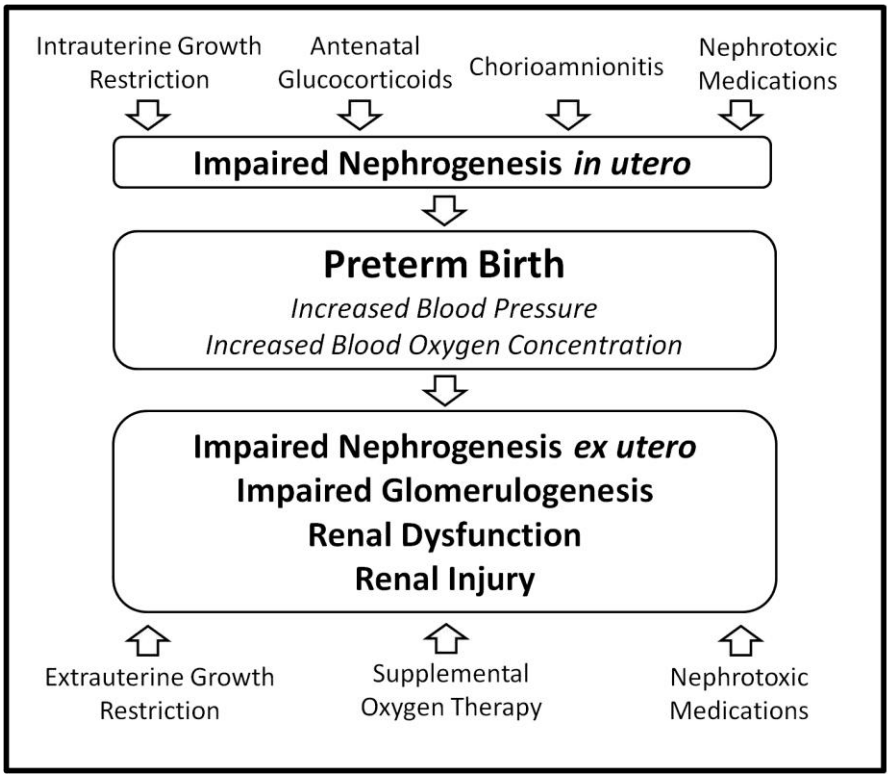

Figure 7.2: Diagram describing antenatal and postnatal factors, and the haemodynamic changes that occur at birth, that may individually or in combination impair nephrogenesis, glomerulogenesis, and/or lead to renal injury in the preterm neonate.

7.2.1 ANTENATAL FACTORS

INTRAUTERINE GROWTH RESTRICTION

Intrauterine growth restriction (IUGR) is defined as fetal growth below the 10th percentile for gestational age; a high proportion of preterm neonates are also growth restricted *in utero*, with severe IUGR often requiring clinical intervention and the induction of preterm delivery (Bamfo and Odibo, 2011). Importantly, IUGR has been strongly linked to a reduced nephron endowment at birth (Hinchliffe *et al.*, 1992; Manalich *et al.*, 2000) as well as renal dysfunction (White *et al.*, 2009); the impairment of renal growth

accompanying IUGR likely relates to the reduced renal blood flow that occurs as a consequence of brain sparing (Behrman *et al.*, 1970). Previous findings from studies in children and adults that were born preterm as well as IUGR, determined that both kidney growth (Drougia *et al.*, 2009) and renal function (Keijzer-Veen *et al.*, 2005) were impaired in comparison to individuals born preterm at an appropriate weight for gestational age

In contrast to these findings, in this thesis there was no evidence to suggest that IUGR adversely affected renal development (Chapter 3). There was no significant difference in kidney weight or indices of renal development (glomerular generation number, nephrogenic zone width, renal corpuscle size) in IUGR preterm neonates compared to preterm neonates that were not growth restricted *in utero* (Sutherland *et al.*, 2011). This may relate to postnatal catch-up growth of the kidney in the preterm infants whilst nephrogenesis is ongoing. Indeed, kidney size is normally a strong determinant of nephron number (Zohdi *et al.*, 2007; Gubhaju *et al.*, 2009; Sutherland *et al.*, 2009). However, it is to be kept in mind that the sample size of the IUGR-affected infants was relatively low, and thus there may have been insufficient power to detect significant differences.

CHORIOAMNIONITIS

Chorioamnionitis is defined as inflammation/bacterial infection of the fetal membranes of the placenta, and it is a very common antecedent of preterm birth (Goldenberg *et al.*, 2000; Menon *et al.*, 2010). Intrauterine infection is known to result in a fetal inflammatory response, with markers of inflammation and tissue injury observed in fetal lung, brain and thymus (Moss *et al.*, 2002; Nitsos *et al.*, 2006; Murthy and Kennea, 2007; Kunzmann *et al.*, 2010). Although it has not been definitively established, it is conceivable that chorioamnionitis would also result in renal inflammation.

Importantly, in a study of renal development in a sheep model of preterm birth, nephron endowment was shown to be significantly reduced (by 23%) following exposure to experimentally-induced chorioamnionitis during late gestation (Galinsky *et al.*, 2011). Studies in human preterm neonates have also shown a significant correlation between intrauterine inflammation and renal dysfunction (Itabashi *et al.*, 2003). The mediators of reduced nephron endowment and renal dysfunction in the preterm kidney following

chorioamnionitis exposure are currently unknown. It may be speculated, however, that localised renal inflammation, a reduction in renal perfusion (Yoon *et al.*, 1999), and/or any adverse effects of chorioamnionitis on overall fetal growth (Menon *et al.*, 2010) may influence renal development in this condition.

The treatment of chorioamnionitis (and other bacterial infections) in pregnant women may necessitate the administration of antibiotics; however, the usefulness of antibiotic treatment in the prevention of infection-induced preterm delivery is uncertain (Goldenberg *et al.*, 2000; Klein and Gibbs, 2004; Hutzal *et al.*, 2008). Maternal antibiotic treatment certainly has the potential to adversely impact on fetal renal development. Experimental studies have shown that antenatal exposure to the most commonly-administered aminoglycoside antibiotic, gentamicin, results in reductions in nephron endowment (Mallie *et al.*, 1986; Gilbert *et al.*, 1987; Gilbert *et al.*, 1990; Gilbert *et al.*, 1994; Cullen *et al.*, 2000); through organ culture experiments, it was determined that the nephron deficit resulting from gentamicin exposure was likely caused by impaired branching morphogenesis (Gilbert *et al.*, 1994; Cullen *et al.*, 2000). Importantly, it has been shown that gentamicin accumulates in renal proximal tubule cells, leading to tubular cell necrosis (Nagai and Takano, 2004). Similarly, exposure of the developing kidney to beta-lactam antibiotics (such as ampicillin) has been demonstrated to result in impaired nephrogenesis, and cystic tubular dilation (Nathanson *et al.*, 2000). Overall, these findings suggest that chorioamnionitis, in association with antenatal antibiotic exposure, may have considerable deleterious effects on fetal renal development.

ANTENATAL GLUCOCORTICOIDS

It is now standard clinical practice for glucocorticoids (betamethasone or dexamethasone) to be administered to pregnant women at risk of preterm delivery; this treatment results in accelerated fetal lung maturation and thereby increases survival rates in preterm neonates (Shanks *et al.*, 2010; Crowther *et al.*, 2011). Certainly, the majority of mothers (79.4%) who were recruited for the neonatal renal function study (having given birth at the Monash Medical Centre between 2008 and 2011) were administered betamethasone prior to preterm delivery (Chapter 2). Importantly, besides the beneficial effects on lung function, antenatal glucocorticoid treatment is also associated with increased mean

arterial blood pressure, and increased renal blood flow and GFR (Stonestreet *et al.*, 1983; al-Dahan *et al.*, 1987; Kari *et al.*, 1994; van den Anker *et al.*, 1994; Ervin *et al.*, 1996; Ervin *et al.*, 1998; Jahnukainen *et al.*, 2001), suggesting that accelerated functional maturation of the kidney may also occur.

In previously published studies performed in animal models (including rodents (Celsi *et al.*, 1998; Ortiz *et al.*, 2001; Ortiz *et al.*, 2003) and sheep (Wintour *et al.*, 2003)), antenatal glucocorticoid administration was shown to lead to a significant reduction in nephron endowment of the exposed offspring. A study by de Vries *et al*. (2010) similarly showed that postnatal administration of dexamethasone in the neonatal rat (at a time of ongoing postnatal nephrogenesis) leads to a reduction in glomerular density. In contrast, however, studies conducted in a baboon model of preterm birth (involving careful replication of the human clinical setting) showed that nephron endowment was not adversely affected by antenatal glucocorticoid exposure (Gubhaju *et al.*, 2009). The disparity in the results of these studies highlights the potential for a differential effect of glucocorticoid exposure on the developing kidney according to the dosage, and also the timing of exposure. Importantly, there was evidence of accelerated renal maturation in the preterm baboon kidneys, with a greater number of mature nephrons present in the glucocorticoid-exposed kidneys compared to those that were not exposed when examined at both the time of caesarean delivery and also at postnatal day 21 (Gubhaju *et al.*, 2009). This result parallels the findings of increased renal functional capacity following antenatal glucocorticoid exposure, and furthermore is consistent with the accelerated renal maturation observed in the human neonatal kidney after preterm birth (Chapter 3; Sutherland *et al*. (2011)).

ANTENATAL TOCOLYTICS

Tocolytic drugs are commonly administered to pregnant women at risk of preterm delivery in order to inhibit uterine contractions and thereby prolong pregnancy (Pryde *et al.*, 2001). One well-known tocolytic is indomethacin, a member of the NSAID class of drugs, which functions by inhibiting cyclooxygenase enzyme activity and thus reducing prostaglandin synthesis (Warner *et al.*, 1999). Antenatal indomethacin administration has been shown to be highly effective at prolonging pregnancy; however, its use is generally

considered to be controversial due to the potential for side-effects in the developing fetus (Pryde *et al.*, 2001). In the kidney, nephron deficits and reduced cortical renal volume (Komhoff *et al.*, 2000; Saez *et al.*, 2009; Olliges *et al.*, 2011) as well as severe renal dysplasia (Norwood *et al.*, 2000) have been reported in experimental models of prostaglandin inhibition. Antenatal exposure to indomethacin has also been associated with glomerular cystic changes coupled with postnatal renal dysfunction in human neonates (Kaplan *et al.*, 1994; van der Heijden *et al.*, 1994). The cystic glomeruli were visibly similar in appearance to the abnormal glomeruli described in this thesis (Chapters 3 and 6); however, as a number of the affected neonates in the previous studies were also born preterm, it is not clear whether the antenatal indomethacin exposure was the cause of the abnormalities.

7.2.2 POSTNATAL FACTORS

HAEMODYNAMIC CHANGES AT BIRTH

The sudden haemodynamic changes that occur at birth as the neonate transitions into the extrauterine environment include a significant increase in renal blood flow (Veille *et al.*, 1998), and also a redistribution of intrarenal blood flow, with the majority of flow shifting from the medulla to the inner renal cortex (Robillard *et al.*, 1981). With increasing age and renal maturity, intrarenal blood flow has been shown to be further redistributed from the inner to the outer renal cortex (Jose *et al.*, 1971; Olbing *et al.*, 1973; Robillard *et al.*, 1981). Ratliff *et al*. (2007) used a newborn porcine model (where nephrogenesis is ongoing following term birth) and demonstrated that the distribution of blood flow within the renal cortex is spatially and temporally regulated by glomerular endothelial nitric oxide synthase (eNOS) expression. Low eNOS expression, within the capillaries of developing glomeruli in the outer renal cortex, appears to direct blood flow away from the nephrogenic zone towards the more mature and functional juxtamedullary glomeruli with higher eNOS expression (Ratliff *et al.*, 2007). The pattern of blood flow distribution in the immature kidney following preterm birth, and whether increased blood flow may be damaging to developing glomerular capillaries is currently unknown; this is an important area for future research.

In the preterm lamb kidney at postnatal day three, significant glomerular capillary dilation occurred following birth (Chapter 4); underlying this structural change may be increases in intraglomerular pressure which result in mechanical stretch of the capillary wall (Kriz and Endlich, 2005). In addition, it is to be noted that in the preterm baboon study (Chapter 6; Sutherland *et al*. (2012)), the highest percentage (and greatest inter-individual range) of abnormal glomeruli per kidney was observed in the group of animals that did not receive NSAID treatment after birth. The majority of these untreated animals required dopamine and hydrocortisone treatments postnatally following persistent hypotension. This is likely to have led to abrupt changes in blood pressure, and thus has the potential to lead to glomerular injury.

HYPEROXIA

The intrauterine environment, which is optimal for renal development (Tufro-McReddie *et al.*, 1997), is relatively hypoxic (Rodesch *et al.*, 1992; Fischer and Bavister, 1993); after normal birth, blood oxygen saturations double within the first five minutes as the neonate commences breathing in the extrauterine environment (Kamlin *et al.*, 2006; Rabi *et al.*, 2006). Given neonatal lung immaturity, however, supplemental oxygen therapy is commonly required in preterm neonates. The infants may be exposed to oxygen concentrations ranging from 21% (room air) up to 100% during their initial resuscitation, and ongoing ventilation (Tan *et al.*, 2005; Rabi *et al.*, 2011). Hyperoxia, and the resulting oxidative stress of the neonate (Vento *et al.*, 2001; Vento *et al.*, 2003), is involved in the pathogenesis of a number of common morbidities of prematurity such as retinopathy of prematurity and bronchopulmonary dysplasia (Saugstad, 2001); underlying each of these conditions is significant impairments in vascular development following the downregulation of VEGF expression (Maniscalco *et al.*, 2005; Fujinaga *et al.*, 2009). As nephrogenesis (and thus renal vascularisation) is often ongoing following preterm delivery, it is likely that development of the glomerular capillaries may also be affected. Recent research in a rodent model has supported this premise, whereby exposure to hyperoxia (80% O_2) in the early postnatal period, at a time when nephrogenesis was ongoing, led to microvascular rarefaction, vascular dysfunction, and a significantly

reduced number of nephrons in rat kidneys examined at 25-35 weeks of age (Yzydorczyk *et al.*, 2008).

Interestingly, however, in the study presented in this thesis (Chapter 5) newborn mice pups exposed to lower oxygen concentrations (65% O_2) for seven days postnatally were not observed to have any impairments in nephrogenesis; accordingly, nephron number in adulthood was within the normal range. The difference in the findings between this study and that of Yzydorczyk *et al*. (2008) may relate to the concentration of oxygen used (80% *versus* 65% O_2), and/or the timing of nephron number assessment (25-35 weeks *versus* 8 weeks). Importantly, in the current study glomerular hypertrophy was observed in the hyperoxia-exposed mice kidneys in adulthood; although nephron number was within the normal range, the glomerular hypertrophy may be indicative of a reduced renal functional capacity in these animals. The cause of this could not be determined in the present study, but the findings do not rule out the possibility of impaired glomerular capillary growth.

Interestingly, morphologically abnormal glomeruli were not observed in either the hyperoxia-exposed mice kidneys (Chapter 5) or lamb kidneys following preterm birth (at a time point when nephrogenesis was already completed; Chapter 4). Taken together, these results suggest that it is glomeruli formed in the extrauterine environment that are most at risk of impaired glomerular development and/or injury, and that postnatal hyperoxia exposure is not the cause of the abnormality. Preterm neonates that are born after the completion of nephrogenesis (normally completed between 32 and 36 weeks gestation) are therefore likely to be largely unaffected.

EARLY POSTNATAL NUTRITION

Extrauterine growth restriction (EUGR) refers to postnatal growth of the preterm infant that is below the 10^{th} percentile of intrauterine growth expectation; in general, preterm neonates do not achieve the same rate of growth postnatally compared to normal growth *in utero* (Clark *et al.*, 2003). Just as IUGR is known to have a significant adverse effect on renal development and function, it is likely that ongoing postnatal nephrogenesis is also influenced by restricted postnatal growth. Indeed, Bachetta *et al*. (2009) demonstrated

that both IUGR and EUGR were associated with reduced GFR in 7 year old children that were born at less than 30 weeks gestation.

Previous research has also highlighted the importance of fetal/neonatal nutritional intake in order to allow for adequate development of the kidney. For example, high protein intakes (such as through the protein supplementation of breast milk) are associated with improvements in overall postnatal growth (Velaphi, 2011). Furthermore, infants fed with protein-rich formula have been observed to have a significantly increased kidney size compared to infants fed with breast milk (Schmidt *et al.*, 2004). Adequate vitamin A (retinoic acid) levels have also been demonstrated to be essential for renal development, in particular during the stage of branching morphogenesis (Vilar *et al.*, 1996; Moreau *et al.*, 1998; Bhat and Manolescu, 2008). In previous studies, a strong correlation between maternal vitamin A status and nephron endowment of rat offspring has been demonstrated (Lelievre-Pegorier *et al.*, 1998), and importantly, a single bolus dosage of retinoic acid during mid-gestation was able to prevent the reduction in nephron endowment expected in rat offspring in a model of maternal protein restriction (Makrakis *et al.*, 2007). In contrast, retinoic acid administration does not appear to enhance nephrogenesis when administered during the period of ongoing postnatal nephrogenesis in the preterm baboon neonate; this is probably because the retinoic acid was administered after the cessation of active branching morphogenesis in these animals (Sutherland *et al.*, 2009).

NEPHROTOXIC MEDICATIONS

The administration of a variety of nephrotoxic medications is common practice in the clinical care of the preterm neonate (Zaffanello *et al.*, 2010; Schreuder *et al.*, 2011). Indeed, from the findings of the renal function study reported in this thesis (Chapter 2) it can be seen that preterm neonates are routinely administered six different classes of antibiotics, antifungal agents, methylxanthines, inotropes, steroids, NSAIDs and diuretics (other drugs, such as morphine, were not listed). Given their immaturity at birth and related adverse health outcomes, neonates born extremely preterm (≤ 28 weeks gestation) are the infants most commonly exposed to these medications (Chapter 2).

Preterm neonates often receive prophylactic antibiotic treatment after preterm birth and/or additional antibiotics in cases of localised infection or sepsis. Of concern, NSAIDs (indomethacin and ibuprofen) and aminoglycoside antibiotics (gentamicin) are known to adversely affect postnatal renal function, as well as lead to renal injury. Therefore, they are considered to be a likely cause of the glomerular abnormalities observed in the preterm kidneys. Aminoglycoside exposure primarily results in renal tubular necrosis (Rodriguez-Barbero *et al.*, 1997), which consequently leads to increased sodium excretion, proteinuria, and a significant reduction in GFR (Langhendries *et al.*, 1988; Giapros *et al.*, 2003; Tugay *et al.*, 2006). NSAIDs, utilised in the treatment of a patent ductus arteriosus (Hermes-DeSantis and Clyman, 2006), are another potential cause of glomerular abnormalities. Indeed, the administration of NSAIDs causes systemic vasoconstriction which in turn leads to significant reductions in renal blood flow and urine output (Kang *et al.*, 1999; Sener and Smith, 2002; Keller *et al.*, 2005). Importantly, the low GFR observed in preterm neonates treated with these medications may also result in excessive renal accumulation of these drugs which imparts a significant risk of acute renal injury. In this regard, an experimental study conducted in a neonatal rodent model demonstrated that postnatal administration of NSAIDs and/or gentamicin during the period of postnatal nephrogenesis is associated with a number of structural changes in the kidney (Kent *et al.*, 2007). These changes included proximal tubule vacuolization, interstitial oedema, and podocyte foot process effacement; the most severe effects were observed in animals that received combined NSAID and gentamicin treatment (Kent *et al.*, 2007).

Although there is mounting evidence linking NSAID treatment with deleterious effects in the kidney, the findings of this thesis (Chapter 6; Sutherland *et al.* (2012)) clearly demonstrate that early postnatal NSAID (ibuprofen) treatment is not the cause of the morphologically abnormal glomeruli in the preterm kidney. Abnormal glomeruli (with enlarged Bowman's space and shrunken glomerular tuft) were observed in the outer renal cortex of the preterm baboon kidneys at a range of 0-22% per kidney in untreated animals, and 0-6% per kidney in those that received ibuprofen treatment after birth.

Although ibuprofen exposure was not the cause of the glomerular abnormalities, it did result in a significant reduction in nephrogenic zone width in the preterm baboon kidney

(Chapter 6; Sutherland *et al.* (2012)). This suggests that prostaglandin inhibition may result in the early cessation of nephrogenesis; however, given the short time period usually recommended for the treatment of patent ductus arteriosus (Ohlsson *et al.*, 2010), the effect on renal development is likely to be minimal. In this regard, Kent *et al*. (2009) demonstrated that there was no effect of early postnatal NSAID and gentamicin exposure on nephron endowment in a rat model.

7.3 CONSEQUENCES OF IMPAIRED RENAL DEVELOPMENT AND RENAL INJURY

The most important indicator of life-long renal functional capacity is nephron number; a low nephron number at the completion of nephrogenesis (low nephron endowment) has been causally linked to the development of renal disease later in life (Hoy *et al.*, 2005). Importantly, recent studies in a mouse model of preterm birth have reported a reduced nephron endowment following preterm delivery (Stelloh *et al.*, 2012), and in human preterm neonates a significantly reduced number of glomerular generations has been reported (Rodriguez *et al.*, 2004). Through the series of studies presented in this thesis, kidneys were only assessed at early postnatal time points (often when nephrogenesis was still ongoing) which did not allow for the determination of final nephron number, or glomerular generation number, following preterm birth. However, there were a number of renal adaptations and/or abnormalities following preterm birth which suggest that the final complement of nephrons does not reach the predicted potential, such as accelerated maturation postnatally (reduced nephrogenic zone width), and high numbers of abnormal glomeruli in some kidneys (it is unlikely that these glomeruli will ever be functional). Given that a reduced nephron endowment is linked to the development of hypertension (Hoy *et al.*, 2005; Ingelfinger, 2008), it is not surprising given the findings of this thesis that there are a number of recent studies reporting an elevation in blood pressure in children (Stevenson *et al.*, 2001; Bonamy *et al.*, 2005; Bonamy *et al.*, 2007) and adults born preterm (Siewert-Delle and Ljungman, 1998; Kistner *et al.*, 2000; Doyle *et al.*, 2003; Hack *et al.*, 2005; Johansson *et al.*, 2005; Keijzer-Veen *et al.*, 2005 ; Keijzer-Veen *et al.*, 2007; Lawlor *et al.*, 2007; Cooper *et al.*, 2009).

Besides the accelerated postnatal maturation and the presence of abnormal glomeruli, a major finding of concern in this thesis was the glomerular hypertrophy observed in the kidneys of the human preterm neonates (Chapter 3) and also in animal models (Chapters 4 and 5). It is to be expected that these renal structural modifications have occurred in order to compensate for the immaturity (and therefore low functional capacity) of the kidney after birth. In the short term, this may be beneficial for the preterm neonate in order to cope with the abrupt increase in renal functional demand. As explained by the *developmental origins of health and disease hypothesis*, however, adaptive organ changes that promote short-term survival may in the long-term predispose the organ to adult disease (Tarry-Adkins and Ozanne, 2011). This hypothesis has been predominantly linked to the consequences of impaired fetal development *in utero*, however it may be argued that it is equally relevant to preterm neonates in whom organogenesis is ongoing postnatally.

It is unknown at this stage, however, whether the initial renal adaptation in preterm infants (which enables an increase in the glomerular capillary surface area available for filtration) will persist later in life, and as such whether it will lead to adverse structural changes in the long-term. If this is the case, it is of major concern. Indeed, glomerular hypertrophy is a key antecedent to renal disease, with glomerular hyperfiltration leading to a sequelae of glomerulosclerosis and subsequent glomerular loss (D'Agati, 2003; Kriz and Endlich, 2005), and thus further reductions in the functional capacity of the kidney. Hence, if glomerular hypertrophy is already present at the beginning of life in subjects born preterm, their kidneys will most certainly be predisposed to long-term renal disease. In this regard, there are some studies to have described renal dysfunction in childhood (Rodriguez-Soriano *et al.*, 2005; Iacobelli *et al.*, 2007) and adulthood following preterm birth (Keijzer-Veen *et al.*, 2005). Additionally, in a small case study, renal pathology was assessed in a group of six people that were born preterm (22-30 weeks gestation) and examined at 15-52 years of age (Hodgin *et al.*, 2009). Typical findings of post-adaptive focal segmental glomerulosclerosis (FSGS) were reported, and in the absence of other risk factors for FSGS in these individuals, the glomerulosclerosis was attributed to preterm birth (Hodgin *et al.*, 2009).

Early life hyperoxia exposure was also shown to result in glomerular hypertrophy in adulthood, independently of preterm birth (Chapter 5). Exposure to hyperoxia in the neonatal period may be an additive insult to the initial induction of hypertrophy due to preterm birth. In this regard, IUGR (Keijzer-Veen *et al.*, 2005), neonatal hypotension, postnatal catch-up growth (Iacobelli *et al.*, 2007) and childhood obesity (Abitbol *et al.*, 2009) have been linked to a greater extent of renal dysfunction in preterm-born individuals, reflecting the importance of additive risk in the progression of renal disease. Differences in the postnatal clinical course of the preterm neonate, and exposure to the various antenatal/postnatal insults will likely result in differences in the extent of the reduction of renal functional capacity following preterm birth. Certainly, the percentage of abnormal glomeruli in the preterm kidney (Chapters 3 and 6), and the levels of urinary protein and NGAL excretion (Chapter 2) was highly variable between individual neonates suggesting that some infants are more adversely affected than others; furthermore, evidence of renal dysfunction in children and adults following preterm birth is not a universal finding (Vanpee *et al.*, 1992; Kistner *et al.*, 2000; Keijzer-Veen *et al.*, 2007; Rakow *et al.*, 2008).

7.4 FUTURE DIRECTIONS

The findings from this series of studies clearly indicate that nephrogenesis is ongoing following preterm birth; however, the kidney is vulnerable to impaired nephrogenesis and also renal injury. In order to build from these findings, in future studies it will be essential to: 1) identify a suitable biomarker for the detection of acute kidney injury in the preterm neonate; 2) further characterise the specific effects of preterm birth and related antenatal and postnatal factors on the developing kidney; 3) characterise the structure of the abnormal glomeruli, and to delineate their pathogenesis; and 4) conduct long-term follow-up studies in order to definitively determine the effects of preterm birth on life-long renal health.

Given the importance of the early diagnosis and treatment of AKI, it is essential that future research be focused on the discovery of novel urinary biomarkers for use in the preterm neonate. The expectation of a new biomarker is to enable the diagnosis of

cellular injury before a decline in renal function occurs; as described by Rosner (2009), it would be optimal if a biomarker could also be utilised to measure the response to, and any adverse effects of, therapeutic interventions, indicate the severity of renal injury, the aetiology of the injury and the location of the injured cells. Parikh *et al.* (2010), in a systematic review of the current literature, determined that the molecules with the most promise for the early diagnosis of AKI in the general human population include interleukin-18 (IL-18), fatty acid binding protein (FABP), cystatin-C, and neutrophil gelatinase-associated lipocalin (NGAL). In the current series of studies, however, it was determined that urinary NGAL levels are not an accurate indicator of renal injury in the preterm neonate (Chapter 2). Future studies, therefore, should be focused on examining the utility of other biomarkers that have been identified as having potential for use in the clinical setting, and that these biomarkers are specifically assessed in a population of preterm neonates.

From the findings reported in this thesis, it was shown that early postnatal ibuprofen administration is associated with a reduced nephrogenic zone width, and neonatal hyperoxia exposure results in glomerular hypertrophy in adulthood; neither of these factors was causative of the morphologically abnormal glomeruli in the preterm kidney. As discussed in this thesis, however, there are numerous other antenatal and postnatal factors associated with preterm birth that have the potential to directly influence renal development and function. Certain factors are inherent to the circumstance of preterm birth (such as the redistribution of blood flow), some relate to medical conditions, and related treatments of the mother during pregnancy (including IUGR, and antibiotic treatments for chorioamnionitis), and others are associated with the postnatal clinical care of the preterm neonate (such as nutritional intake and exposure to nephrotoxic medications). To examine each of these factors, it would be ideal for a clinically relevant model of preterm birth (such as the baboon) to be utilised; studies would comprise a comprehensive functional and stereological assessment of renal development in a group of animals that were exposed to the specific factor, compared to unexposed controls. Due to the complexities involved in the treatment of preterm neonates after birth, and the variability in individual treatment responses, it is often not possible for an 'unexposed' group to survive, even in animal models. Small animal models (such as the

rodent) are useful under these circumstances; nephrogenesis is ongoing for 1-2 weeks after normal term birth in mice and rats which makes them ideal models to examine the effects of postnatal insults on ongoing renal development.

Another important focus of future research will be to fully characterise the morphologically abnormal glomeruli that were commonly present in the outer renal cortex of the preterm kidney. To definitively determine whether the glomeruli are atubular, serial sections of affected glomeruli in preterm baboon and human kidney tissue may be examined in order to visually observe the presence or absence of a connection between the proximal tubule and the Bowman's space of the glomerulus. Previous immunohistochemical analyses of abnormal glomeruli in the preterm baboon kidney demonstrated that the shrunken glomerular tuft appears to be composed primarily of podocytes, and may be poorly vascularised (Gubhaju *et al.*, 2009; Sutherland *et al.*, 2009). Immunohistochemical techniques, in conjunction with confocal imaging, could be further utilised to quantify the proportion of different cell types within the glomerular tuft, to assess the capacity for glomerular vascularisation (VEGF expression), and also to determine the proportions of glomerular cells undergoing apoptosis (caspase-3 expression). The cellular composition of affected glomeruli may give an indication as to the pathogenesis of the glomerular abnormalities; a depleted number of endothelial cells, for example, may be indicative of impaired vascular development and/or injury.

It is to date unclear what consequences preterm birth (and related antenatal and postnatal factors) will have on the long-term renal health of individuals born preterm; given the advances in clinical care practices over the past two decades, the first survivors of extremely preterm birth are only now reaching early adulthood. As reported in this thesis, nephrogenesis was still ongoing in the majority of human preterm neonates studied, therefore it was not possible to determine whether there was an effect of preterm birth on the final complement of nephrons formed (Chapter 3; Sutherland *et al.* (2011)). However, the findings are certainly suggestive that there is a negative impact on nephron endowment. Ideally whole kidneys, or a known proportion of the kidney, could be collected at autopsy from older infants (in whom nephrogenesis had ceased), in order to stereologically determine whether preterm birth is associated with a reduced nephron endowment. The kidneys from older children and adults that were born preterm could

also be assessed in order to investigate the possibility of age or injury-related nephron loss at a later time point, and importantly, to determine whether the glomerular hypertrophy observed in the neonatal kidney persists into later life. Periodic Acid Schiff (PAS)-stained sections of kidney tissue from these subjects may also be assessed in order to determine the presence or absence of glomerulosclerosis. If medical records are available for these subjects, it may be possible to perform logistic regression analyses in order to determine if there is a relationship between deficits in nephron number and/or the severity of renal pathology with exposure to specific antenatal or postnatal factors that may have influenced renal development. Due to a low number of subjects, it was unfortunately not possible for us to perform these analyses in either the neonatal autopsy study (Chapter 3) or the renal function study (Chapter 2) presented in the current thesis.

Overall, by identifying the individual factors involved the impaired nephrogenesis and/or injury in the preterm kidney, and by delineating their specific effects, it may be possible in the future for the clinical care practices in the NICU to be modified in order to optimise postnatal renal development.

7.5 CONCLUSIONS

Encouragingly, we have shown through this series of studies that nephrogenesis continues postnatally in the extrauterine environment after preterm birth. Although the functional capacity of the kidney was lower in preterm neonates than in neonates born at term, only a small proportion of neonates exhibited severe renal dysfunction during the first month of life. Of concern, however, pathological proteinuria and high urinary NGAL levels may be indicative of postnatal renal injury. Similarly, morphologically abnormal glomeruli, with shrunken glomerular tufts, were localised to the outer renal cortex of the preterm kidney suggesting that immature glomeruli are at risk of impaired development and/or renal injury following preterm birth. Overall, the presence of non-functional glomeruli, and underlying changes in glomerular capillary size and nephrogenic zone width, may ultimately result in a deficit of functional nephrons in the preterm kidney. In turn, this is likely to have an adverse effect on the short and long-term renal health of

individuals born preterm. It is essential, therefore, that future research focuses on the identification of factors that adversely influence the development of the preterm kidney, in order to develop strategies that can be implemented in the neonatal intensive care setting to optimise postnatal renal development.

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ADDENDA

CHAPTER TWO ADDENDUM

Page 133, add to the end of the 'Impaired renal function' section of the Results:

The relationship between the six different measures of renal dysfunction (Figure 7), and whether any preterm neonates had multiple measures of renal impairment, was also examined. As shown in Table 3, 11 preterm neonates (10.3%) had 2 measures of renal dysfunction, and 1 preterm neonate (0.9%) had 4 measures of renal dysfunction. The categories of renal dysfunction in these 12 individual neonates are described in Table 4. The most common combination of renal dysfunction measures exhibited during the first month of life was high serum creatinine and high fractional excretion of sodium (observed in 7/12 preterm neonates).

Table 3: The number and percentage of preterm neonates (n=107) that had 0 to 6 measures of renal dysfunction (low urine output, low creatinine clearance, high serum creatinine, high fractional excretion of sodium, high urine total protein, and high urinary NGAL).

Number of Measures	Number of Neonates	Percentage of Neonates
0	64	59.8
1	31	28.9
2	11	10.3
3	0	0.0
4	1	0.9
5	0	0.0
6	0	0.0

Table 4: Gestational age group, sex, and multiple measures of renal dysfunction in 12 preterm neonates.

	Gestational Age Group	Sex	Low Urine Output	Low C_{Cr}	High Serum Cr	High FE_{Na}	High UTP	High NGAL
1	A	F	✓		✓	✓		✓
2	A	F			✓	✓		
3	A	F			✓	✓		
4	A	M			✓	✓		
5	B	M			✓	✓		
6	C	M			✓	✓		
7	C	F			✓	✓		
8	A	M	✓			✓		
9	B	F	✓			✓		
10	B	M	✓					✓
11	C	M			✓			✓
12	B	M		✓			✓	

CHAPTER TWO ADDENDUM

Page 138, add prior to 'Urinary NGAL as a marker of acute kidney injury' section of the Discussion:

Measures of renal impairment

In this study, renal impairment was defined as measures of renal function that were greater than 2 standard deviations from the mean (including low urine output, low creatinine clearance, high serum creatinine, high fractional excretion of sodium, high urine total protein, and high urinary NGAL). A limitation of the current study, however, is that it is not clear whether the measures of renal dysfunction we have employed are resultant and/or causative of renal injury. There is a current lack of definition in the literature as to what constitutes acute kidney injury (AKI) in the preterm neonate; if the general definition of AKI commonly used in adults (RIFLE criteria (69, 72)) was applied in the current study, only 1 preterm neonate (and 10 term neonates presenting with oliguria) clearly met the criteria. Therefore, we adopted a broader definition of dysfunction in the current study in order to give an indication as to the percentage of infants with reduced renal functional capacity (glomerular and tubular), rather than focus on the strict AKI criteria. It is important to note that of all the maternal and neonatal factors we examined (including exposure to medications) there was no direct corollary with renal impairment, indicating that the observed renal dysfunction is likely multi-factorial in origin.

A high proportion of preterm neonates were observed to have high FE_{Na}; it may be speculated that the high sodium excretion in these neonates represents either tubular immaturity or injury. Of the neonates that exhibited more than one measure of renal function, the majority had both high FE_{Na} and high serum creatinine. If tubular function is impaired, this may also have an impact on serum creatinine levels as creatinine (besides predominantly being filtered by the glomeruli) is known to be actively excreted by proximal tubular cells (70, 73), and in the case of preterm neonates, may also be reabsorbed by the immature tubules as has been observed in a neonatal animal model (71). Serum creatinine levels are also influenced by extrarenal factors such as muscle mass, and the intake of nitrogen, protein and creatinine (70, 73) which is increased with milk formula and other parenteral nutrition preparations (74). Each of these factors may have influenced the relatively high proportion of neonates who also exhibited high serum creatinine levels; in contrast, only 1 preterm neonate was found to have low creatinine clearance. In general, creatinine clearance is considered to be a more reliable indicator of glomerular filtration rate than serum creatinine levels alone (55), especially given the large number of factors that may influence the generation of creatinine as described above. Encouragingly, this finding may indicate that the capacity for glomerular filtration in the preterm kidney in the early neonatal period is quite adequate; however, tubular function is likely impaired.

Page 144, add to end of the References:

69. **Bellomo R, Ronco C, Kellum JA, Mehta RL, and Palevsky P**. Acute renal failure - definition, outcome measures, animal models, fluid therapy and information technology needs: the Second International Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group. *Crit Care* 8: R204-212, 2004.

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72. **Mehta RL, Kellum JA, Shah SV, Molitoris BA, Ronco C, Warnock DG, and Levin A**. Acute Kidney Injury Network: report of an initiative to improve outcomes in acute kidney injury. *Crit Care* 11: R31, 2007.

73. **Perrone RD, Madias NE, and Levey AS**. Serum creatinine as an index of renal function: new insights into old concepts. *Clin Chem* 38: 1933-1953, 1992.

74. **Premji SS, Fenton TR, and Sauve RS**. Higher versus lower protein intake in formula-fed low birth weight infants. *Cochrane Database Syst Rev* CD003959, 2006.

CHAPTER FOUR ADDENDUM

Page 167, add to the end of the 'Capillary length and surface area density' section of the Results:

As shown in Figure 5, there was a significant positive linear correlation between capillary surface area density and capillary length density in the term kidney, but no correlation was evident in the preterm kidney indicating that capillary surface area was independent of capillary length.

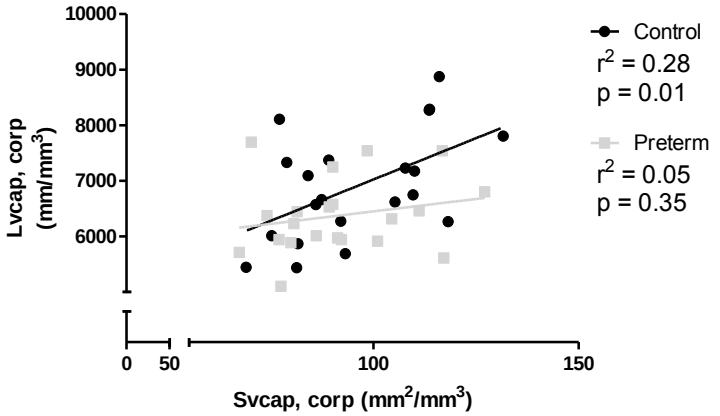

Figure 5: Linear regression analysis of capillary length density per renal corpuscle versus capillary surface area per renal corpuscle in term control (black, circles) and preterm groups (grey, squares). There was a significant positive correlation in the control group, but no correlation in the preterm group.

Page 170, add to first paragraph of 'Preterm birth leads to the dilation of glomerular capillaries' section of the Discussion:

Comment: In support of the conclusion that capillary dilation has occurred in the preterm kidney following preterm birth, we found no correlation between capillary length density and capillary surface area density in the preterm kidney.

CHAPTER FIVE ADDENDUM

Page 192, add to the end of the 'Renal corpuscle volume' section of the Results:

Univariate regression analyses were performed (using Intercooled Stata version 8.0 for Windows statistical analysis software) to determine the effect of the variables hyperoxia exposure, sex and nephron number on renal corpuscle volume. As shown in Table 1, the results of this analysis demonstrated that both exposure to hyperoxia, and nephron number, were independently associated with renal corpuscle volume. There was no association between sex and renal corpuscle volume.

Table 1: Results of univariate analyses to determine the effects of sex, hyperoxia exposure, and nephron number, on renal corpuscle volume in mice at P56.

Variable	Correlation Coefficient (±SE)	R^2	95% Confidence Interval	p value
Sex	$1.45 \times 10^{-6} \pm 2.93 \times 10^{-6}$	0.01	$3.46 \times 10^{-6} – 6.35 \times 10^{-6}$	0.55
Hyperoxia Exposure	$5.08 \times 10^{-6} \pm 2.19 \times 10^{-6}$	0.17	$5.88 \times 10^{-7} – 9.58 \times 10^{-6}$	0.03
Nephron Number	$-3.00 \times 10^{-9} \pm 6.02 \times 10^{-10}$	0.49	$4.24 \times 10^{-9} – 1.76 \times 10^{-9}$	< 0.0001

Page 194, add to first paragraph of 'Induction of glomerular hypertrophy by adulthood' section of the Discussion:

Comment: In support of the finding that renal corpuscle size was significantly increased in the hyperoxia-exposed animals at P56 compared to controls, univariate analysis demonstrated a significant association between hyperoxia exposure and renal corpuscle size.

CHAPTER SEVEN ADDENDUM

Page 229, add to the end of the 'Effects of preterm birth on renal morphology and function' section of the Discussion:

Strengths and limitations of the study design

This thesis incorporated the findings of assessments of renal structural and functional development in human preterm neonates, as well as in neonatal animal (mouse, sheep and baboon) models. The principal strength of the overall study design is the comprehensive assessment of renal function (Chapter 2) and morphology (Chapter 3) in human preterm neonates. The observational study of renal function in preterm neonates being cared for in the NICU setting (described in Chapter 2) was carried out using non-invasive methods, which resulted in high rate of patient participation in the study and clinically relevant results. A weakness of the study, however, was the low number of spot urine samples obtained which possibly limited our ability to fully describe the pattern of urinary protein excretion in preterm neonates during the first month of life. The morphology of the preterm kidney after birth was also assessed in human kidney tissue collected at autopsy (Chapter 3). Very few studies have been previously conducted to examine the effects of preterm birth on kidney development, and a strength of this study was in the separate analysis of neonates that were also IUGR. All preterm neonates examined had died at differing time-points after birth (a range of 2-68 days), and were exposed to a range of factors in the postnatal environment that have the potential to have influenced kidney development. It is possible, therefore, that this intrinsically ill, non-surviving population of preterm neonates are not representative of the population of preterm infants that survive the neonatal period, and may therefore have experienced greater impairments on renal development. The primary limitation of conducting studies in human neonates is that due to the large number of variables, and low sample size, we were not able to perform multivariate analyses in order to determine which factors in the postnatal environment may have contributed to the observed impairments in renal morphology and function. We did not observe any direct correlations between any maternal or neonatal factors (including exposure to medications), therefore it is likely that the impairments are multi-factorial in origin.

In order to eliminate confounding factors, experimental studies (reported in chapters 4, 5, and 6) were conducted in carefully controlled animal models. A sheep model of preterm birth (Chapter 4) was used for the specific assessment of capillary growth in the first 3 days after birth. Although nephrogenesis is completed prior to preterm delivery in this model, vascularisation of the immature glomeruli is likely still ongoing. A weakness of this study was the lack of an age-matched control for comparison with the preterm group; however, the preterm lambs are delivered before lung maturation is complete and therefore they are unable to survive postnatally without ventilation. Fetal animals, matched for post-conceptional age, would be the only option for a control group in this case. In the assessment of the effects of hyperoxia exposure on the developing kidney, a neonatal mouse model followed out to adulthood was utilised (Chapter 5). In rodents, nephrogenesis normally continues in the postnatal environment after birth, which enables the assessment of the effects of postnatal insults on the kidney, and makes them a good model of the preterm neonate. The oxygen concentrations utilised in this study (65% O_2 from day 0-7 of life) represents a relatively high oxygen concentration for a prolonged period of exposure; this may not accurately reflect the clinical setting in which a variable concentration of oxygen may be used, and with more intermittent periods of exposure. The highly relevant baboon model of preterm birth was also utilised in this thesis (Chapter 6), in order to determine the effects of postnatal ibuprofen administration on kidney development. The ontogeny of the baboon kidney very closely resembles that of humans, with ongoing nephrogenesis after preterm birth, which makes the baboon an excellent model. In this study, the administration of both ibuprofen and a NOS inhibitor were examined. The examination of the effects of ibuprofen treatment on the kidney has important clinical relevance as it is a drug commonly used in clinical practice to treat PDA in preterm neonates. In contrast, the administration of NOS inhibitors in preterm neonates is purely an experimental procedure, and its use has only been previously reported in a single clinical trial (as it has been withdrawn from clinical use due to adverse effects).